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A STATISTICAL STUDY OF GENERIC CHARACTERS IN THE COCCACEÆ.*

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ASSISTED BY

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I. PURPOSE OF THE INVESTIGATION.

There has been placed in the hands of the biologist within the last few years a new instrument of research of the highest value. This is the statistical method, first suggested for the study of human characteristics by Quetelet (1846), specifically applied to the bio-

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logical problems of variation and heredity by Galton (1889), and extended and developed in detail by Pearson and his pupils. The most important papers on this subject may be found in the files of the *Philosophical Transactions of the Royal Society of London* and in *Biometrika*. Admirable brief summaries have been prepared by Pearson (1900) and Bigelow (1904).

In many fields of science the statistical method, in its strict sense, is not applicable. Where laboratory experiments may be made, as in most fields of physics and chemistry, a comparatively small array of data obtained under perfectly controlled conditions may permit the derivation of laws of relationship without extensive statistical analysis. The same thing is true in certain fields of biological research. As soon, however, as we proceed to the subtler problems of evolution, it becomes necessary to accumulate a large number of observations and to analyze them by recognized statistical methods. These methods alone have brought order out of chaos in anthropology (Ripley, 1899). They have laid the first foundation for a real science of mental and social phenomena (Thorndike, 1904; Woods, 1906). They offer the most promising clue for tracing the true relationships among the lower forms of plant and animal life.

As we have elsewhere pointed out, the classification of the bacteria presents peculiar difficulties.

> Morphological distinctions are so slight that physiological characters must necessarily be invoked in order to separate and classify the various organisms, and these physiological characters are often variable. Pathogenicity may be taken as a type of those powers of the organism which are easily and profoundly modified by external conditions. On the other hand, there are numerous characters which appear to be extremely constant. Such minute differences as occur in the resistance of different races to unfavorable conditions often remain unchanged through long periods of cultivation. In using these constant characters for classification we are met by another difficulty. Though constant, the differences are very minute, and in studying a number of organisms a perfect gradation is often found between the widest extremes. This is exactly what should be expected from organisms which reproduce only by asexual methods, since it is the fusion of independent cells which swamps minor differences producing the uniformity of species among higher plants. With asexual reproduction every minute variation which is inheritable must persist unchanged until some other chance variation occurs. Each such variation means a new and different type of bacterium.
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> The immense number of generations which may succeed each other in a short space of time makes boundary lines as shifting as they would become among the higher plants if a dozen geological epochs were considered all at once.
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> Since with unicellular organisms acquired characters may probably be inherited in a higher degree than with other forms, existing races of bacteria will be markedly

influenced by the selective effect of environmental conditions, and must bear the impress of their recent history.

There are, therefore, no species among the bacteria in quite the sense in which we ordinarily use the word—as indicating a group of individuals bound together by a number of constant characters and easily identified by mutual fertility. From one point of view each distinct race might be considered a species; but to apply a name for every grade of difference in each varying character would be impracticable; and such names could have no true specific value. The best solution of the difficulty is the establishment of certain types around which the original organisms may be more or less closely grouped; but it must be clearly recognized that the groups thus formed are defined by relation to the type at their center and are not sharply marked off at their extremities from the other groups adjacent.[1]

For these reasons the science of systematic bacteriology has remained in a notably undeveloped state. A score of large groups of bacteria have been more or less satisfactorily recognized by Flügge (1896) and others. Certain of these groups, like the aërobic spore-formers, the colon bacilli, and the diphtheria bacilli, doubtless represent true natural families or genera. In one such group, that of the aërobic spore-formers, where appreciable morphological differences exist, the species and varieties have been carefully worked out by Chester (1904). Far too many specific names among the bacteria however, mean less than nothing. The incomplete description of a vast number of identical or minutely differing forms has led to a confusion quite disheartening to the student of such systematic works as those of Migula (1900) and Chester (1901). Among the Coccaceæ we have compared the published descriptions of 445 species and found evidence for only 31 distinct types (Winslow and Rogers, 1905). These are defined mainly by arbitrary combinations of the three characters of acid production, chromogenesis, and the liquefaction of gelatin. It is small wonder that most bacteriologists have abandoned any attempt at a natural classification, and have sought refuge in such frankly arbitrary schematic groupings as those of Fuller and Johnson (1899), Weston and Kendall (1902), and Jordan (1903). The same tendency carried to its extreme is shown in the decimal systems of Gage and Phelps (1903), and Kendall (1903), and in the modifications recently adopted by the Society of American Bacteriologists.

These systems are most valuable for a routine descriptive work, and for arranging and cataloguing records of cultures. They may, however, lead to error, unless used with due caution. In the first

[1] WINSLOW AND ROGERS, 1905

place, the determinations on which such schemes are based are usually qualitative only and not quantitative. In the second place, the application to all bacteria of one fixed series of characters arranged in an arbitrary order tends to suggest a mechanical view of bacterial relationships which is very far from the complex truth.

In order to obtain a just idea of the real relations of organisms, it is necessary to consider each systematic group by itself. As Robinson has pointed out in an admirable paper on generic classification (Robinson, 1906), "a difference having great classificatory significance in one place may be almost valueless in another." In studying any one group it is therefore necessary to examine afresh each of the various characters used for the identification of bacteria in general, and to determine its local value and significance. Secondly, under each character it is necessary to determine how many distinct types of structure or function may occur. This can be done only by measuring the character quantitatively in a large series of individuals, and plotting curves of frequency which will show whether the individual forms fluctuate about one or several modes. This has been attempted by Howe (1904) with good results, for the composition of the gas produced in dextrose broth by organisms of the *B. coli* group.

Finally, the correlation between various properties should be determined, since it is obvious that the presence of several distinct characters in association is generally of more significance in classification than that of any one alone.

In the present study we have attempted to obtain the data indicated, for certain groups of the Coccaceæ. We have measured the easily and definitely measurable, variable characters in 500 separately isolated races of organisms, and analyzed the data obtained, with two ends in view. We have first plotted the frequency curve for each character to find whether the array varies about one or several modes, and where the modes are situated, with some measure of the extent of variation about these centers. In the second place, we have calculated correlation factors for the most significant pairs of characters. Each mode on the curves of frequency may fairly be taken to mark a natural species or variety, and the characters which vary together must form the most important basis for the establishment of the larger groups. By such a method alone it is

possible to locate those mountain peaks in the chain of bacterial variations which rightly deserve generic and specific names, although records of the characters of individual races by the decimal system are of the greatest value in mapping out intermediate regions. Only the statistical study of numerous individuals by comparable quantitative methods can reveal the general laws of natural classification among the bacteria; and this study must be made in each group with an open mind free from arbitrary predispositions.

We desire in advance to deprecate a comparison between the present work and the numerous detailed and exact biometrical studies which have appeared in other fields. In bacteriology our methods of measurement are crude and tedious, and the general knowledge requisite for the selection of a homogeneous mass of material is lacking. We should know the outlines of the general groups of the cocci, for example, before we can properly select material to study variation in any one of them.

II. METHODS OF THE INVESTIGATION.

1. ISOLATION OF CULTURES.

With regard to the larger groups of the Coccaceæ we have elsewhere shown (Winslow and Rogers, 1905) that the family could be divided into two subfamilies and five genera, defined as follows:

Subfamily 1, Paracoccaceæ (Winslow and Rogers): Parasites (thriving only, or best, on, or in, the animal body). Thrive well under anaërobic conditions. Many forms fail to grow on artificial media; none produce abundant surface growths. Planes of fission generally parallel, producing pairs, or short or long chains.

Genus 1, *Diplococcus* (Weichselbaum): Strict parasites. Not growing, or growing very poorly, on artificial media. Cells normally in pairs surrounded by a capsule.

Genus 2, *Streptococcus* (Billroth): Parasites (see above). Cells normally in short or long chains (under unfavorable cultural conditions, sometimes in pairs and small groups, never in large groups or packets). On agar streak effused, translucent growth, often with isolated colonies. In stab culture little surface growth. Sugars fermented with formation of acid.

Subfamily 2, Metacoccaceæ (Winslow and Rogers): Facultative parasites or saprophytes. Thrive best under aërobic conditions.

Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cells aggregated in groups, packets, or zoöglea masses.

Genus 3, *Micrococcus* (Hallier) Cohn: Facultative parasites or saprophytes. Cells in plates or irregular masses (never in long chains or packets). Acid production variable.

Genus 4, *Sarcina* (Goodsir): Saprophytes or facultative parasites. Division under favorable conditions in three places, producing regular packets. Sugars as a rule not fermented.

Genus 5, *Ascococcus* (Cohn): Generally saprophytic and cells imbedded in large, irregularly lobed masses of zoöglea, in process of carbohydrates. Acid usually formed.

In the present investigation we have included representatives of only three of these genera. The organisms belonging to the genus *Diplococcus* do not lend themselves to comparative study on account of the difficulty with which they may be cultivated, and representatives of the genus *Ascococcus* occur, if at all, only in certain peculiar habitats. We have limited our study to forms which can be found in ordinary environments, and which may be cultivated on ordinary laboratory media; that is, to the genera *Streptococcus*, *Micrococcus*, and *Sarcina*.

We have procured our cultures in approximately equal proportions from five different sources: from the internal tissues of the diseased human body, from the outer surfaces of the normal human body, from water, from earth, and from air. Cultures classed under Habitat I, the tissues of the diseased body, were obtained chiefly from the Boston City Hospital, and the Massachusetts General Hospital, of Boston, and the Johns Hopkins Hospital, of Baltimore. We desire to express our cordial thanks to the bacteriologists of these institutions for their courtesy in furnishing us with these organisms. The cultures classed under Habitat II, surfaces of the normal body, were obtained from three sources. A considerable number were isolated from serum tubes, received by the Boston Board of Health for diphtheria diagnosis. In this connection we desire to acknowledge the courtesy of the bacteriologists of the Board. Only those cultures which gave a negative diagnosis for diphtheria were used. Another series of cocci was isolated from the hands of students

in the Massachusetts Institute of Technology. In collecting them each subject rubbed the front and back of one hand with a wet wad of sterile cotton, running the wash water into a sterile cup. Finally a small number of cultures were obtained from excreta of man and animals. Under Habitat III cultures were obtained from a wide variety of natural waters—public supplies, streams, ponds, pools, shallow wells, driven wells, and the sea. Samples were taken as far as possible only from sources held to be free from pollution. Under Habitat IV organisms were isolated from various samples of earth, loam, clay, sand, etc., obtained mainly in different regions of eastern Massachusetts. The cultures grouped under Habitat V were taken from plates exposed to the air, indoor and out, and here are also included certain organisms of unknown origin which appeared as contaminations, or for whose previous history we have no record.

In each case the sample to be studied was first plated on agar and incubated at 20°. Colonies which looked like cocci (not possessing, that is, the characters of such well-marked forms as *B. mesentericus*, *B. Zopfi*, or *B. fluorescens*) were fished to agar streaks; from each sample only one culture was taken, unless several distinct types of colonies appeared. The agar streak cultures were examined under the microscope and, if apparently cocci, were replated in order to insure their purity, again transferred to agar streaks, and again examined under the microscope. All this preliminary work was carried out at 20°, and the stock cultures finally obtained were kept on agar at the same temperature. There can be no doubt that by this method of procedure we failed to obtain many of the more strictly parasitic streptococci which grew only feebly on solid media and are most active at a temperature of 37°. This fact must be taken into account in interpreting our results. For *Micrococcus* and *Sarcina*, however, the series should be fairly representative.

2. SELECTION OF CHARACTERS FOR STUDY.

The characters ordinarily used in descriptive bacteriology are few, particularly in a group of such simple morphology and limited biochemical powers as the Coccaceæ. This number must be still further reduced, however, when we come to inquire which of them really indicate constant and independent variations. In the first place, it

is necessary to eliminate properites which are due mainly to the character of the medium and the conditions of incubation. As we shall show later, those minute differences in the appearance of colonies on gelatin which form the basis for a large number of German descriptions, fall mainly under this head. Secondly, many characters, while really belonging to the organism itself at a given moment, are so easily modified by cultivation under other conditions as to be practically worthless in systematic work. Among the cocci, pathogenicity is a property of this sort. In the third place, it is evidently unfair to give independent weight to characters which are simply the indirect result of other properties already recorded. Thus among the cocci differences in broth cultures are closely connected with the size of the cell aggregates. Organisms growing in large groups, like most of the sarcinæ, produce heavy sediment and often colony-like groups on the walls of the tube, while those in which the cells readily separate exhibit a more diffuse turbidity. Plate cultures add little more information than may be obtained by a careful scrutiny of stabs and streaks; and the growth on potato and blood serum in many groups of bacteria, and particularly among the cocci, are only valuable as measures of that extremely fugitive quality, the general vigor of the culture.

The considerations which have influenced us in the selection of characters for study among the Coccaceæ may be conveniently arranged in the order, and under the headings, of the Report of the Committee on Standard Methods of Water Analysis to the Laboratory Section of the American Public Health Association (1905).

3. MORPHOLOGICAL CHARACTERS.

Form.—The form of the individual cell furnishes no help in the classification of the Coccaceæ, since under favorable conditions all appear as regular spheres. Irregular oval forms occur at times, particularly in cultures freshly isolated from the throat or alimentary tract, but the form usually becomes normal after cultivation.

Manner of grouping.—The grouping of the cell elements offers a character of considerable importance among these bacteria. While the cocci do not exhibit an entirely unchanging form of grouping, the individuals do show a distinct tendency to occur in one of four forms—either in pairs, chains, masses, or packets.

The grouping is somewhat influenced by the age of the culture and by the kind of medium on which it has grown. Even the same culture will show wide variation from the typical arrangement of the elements. For instance, streptococci occur singly, in pairs, chains, and small masses; but the most frequent arrangement, and that obtained under the most favorable conditions (in liquid media), is in chains. Again, sarcinæ occur singly, in pairs, and in small masses as well as in packets, yet the typical form is the sarcina-packet. Cocci grown on Nährstoff regularly occur in plates, and usually capsulated ones.

In a number of preliminary studies we compared the groupings of the same cultures in various media and under various conditions, examining cultures of different ages, from nutrient broth, sugar broth, peptone solution, hay infusions, nutrient agar, and gelatin, and acid and alkaline gelatin. Cultures more than two weeks old showed abnormalities both in the individual cell and in its groupings. With this exception, the differences produced were very slight. The only constant effect of the medium upon grouping which was apparent was a more distinct development of chains in liquid cultures. Organisms which appear as long chains in fresh broth cultures may show only short chains with irregular groups on solid media. In the present study we have omitted the broth morphology for lack of time, and have recorded the grouping only as apparent on the agar streak.

The streaks used were never more than three days old, and the grouping was observed after staining lightly with methylene blue and mounting in cedar oil. Too heavy staining may introduce a serious error by making packets of small sarcinæ appear like large single cells. These observations on the culture stained with methylene blue were controlled by careful observations of the slides prepared for the study of the Gram stain, as noted later.

We have distinguished two main groupings only by this method of examination. The occurrence of packets marks one, and the absence of packets the other, group. In the first group occur the streptococci, which produce pairs, long chains, and irregular groups; and the micrococci, which show pairs, short chains, fours, and irregular groups; while the sarcinæ include organisms which produce fours, irregular groups, and packets, as well as those

extreme forms which show only packets. None of these differences but that between the presence and absence of packets appear on agar with sufficient constancy to be determined definitely. For distinction between streptococci and micrococci the observation of broth cultures would perhaps be valuable.

Dimensions.—The cocci exhibit a range in size from 0.1 to 2.0 μ with considerable variation between individual cells in the same culture. We were somewhat surprised to find that we could demonstrate no definite relation between size and the age of cultures, or the conditions of cultivation. In a series of preliminary studies the same organism was grown on seven kinds of media and examined at intervals during a period of two months. The maximum size, in different cultures, was recorded on the first, second, seventh, 14th, 42d days, and after two months respectively. The maximum size developing in the different kinds of media during those two months was found, respectively, in broth at 37°, broth at 20°, Nährstoff-Heyden, nutrient gelatin, acid and alkaline gelatin, and under anaërobic conditions. In other words, the age and kind of medium had no constant effect, except that in most cases the Nährstoff and other poor media showed the smallest individuals. No

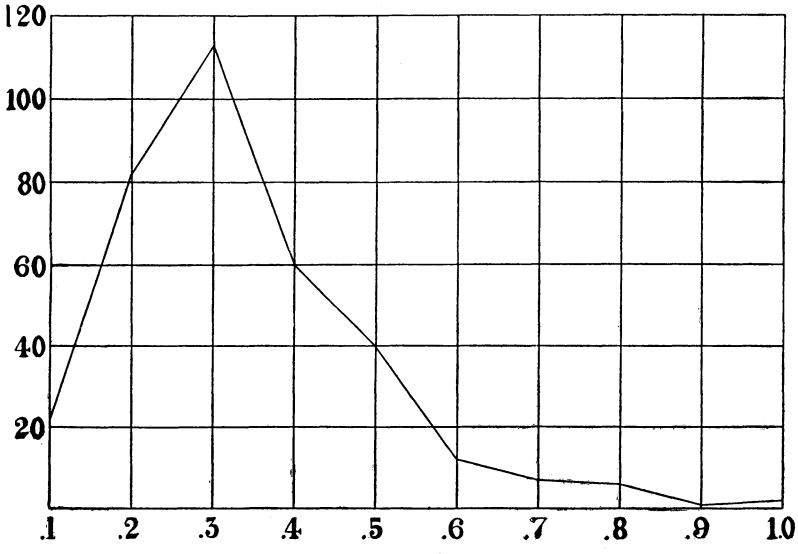

FIG. 1.—Dimensions of 345 cocci. Abscissæ, average diameter in μ. Ordinates, number of cultures.

constant difference in size was apparent in comparing solid and liquid cultures. One series of organisms examined in dextrose broth and on agar, at periods ranging from one day to two weeks, showed the same average size in both media and at all ages. Finally we attempted to see whether prolonged cultivation under special conditions would affect the size of the cell. Cultures were grown for 10 days, in broth at 37°, on nutrient gelatin, and on acid and anaërobic gelatin with daily transfers. The size of each culture was recorded on the 10th day, after which time each was transferred to gelatin and examined after one day. The results showed practically no significant differences.

In a comparison of the size as determined by examination of living organisms and of stained preparations, the cells appeared generally somewhat smaller after staining. This is no doubt partly due to some shrinkage in drying, and partly to the imperfect definition which makes the unstained specimens appear larger than they really are. Occasionally, when the staining was too heavy, the stained cells appeared larger. In any case the differences are unimportant, and we have used the size of the methylene-blue-stained preparation throughout our work.

Staining reactions. — Since the cocci, as far as we have examined them, all stain easily with methylene blue, we have made no special tests with anilin-gentian-violet. The Gram stain has, however, been used on all our cultures, since, in the genus *Diplococcus* and in many other groups, it has been thought to have such special importance.

The value of this staining method has been studied with considerable care by Mr. A. T. Brant, working in the laboratories of the Institute. Mr. Brant found, as other observers have done, that while certain bacteria are constantly Gram-negative or Gram-positive, others exhibit an intermediate condition, retaining the stain under some conditions and giving it up under others. In his, as yet unpublished, paper he notes, for example, that all cultures of *B. coli* are decolorized by one minute's treatment with alcohol, while *B. megatherium* constantly fails to decolorize after three hours. On the other hand, with *B. fluorescens*, *M. pyogenes*, *M. aureus*, and *B. diphtheriae* the result is affected by the time of decolorization, as well as by the age of the cultures. Between the fixed points at the

extreme, preparations will yield varying results, showing some cells stained and others decolorized. As a rule, the large majority of cells in a given preparation will show one reaction or the other; but a second slide made from a similar doubtful case might yield a different result.

The time chosen for decolorization is, of course, an arbitrary factor which will affect the proportion of positive results obtained. In our work, as a result of Mr. Brant's experiments, we fixed on three minutes, although we are not certain that this is really preferable to the five-minute period fixed by the Committee on Standard Methods. We have applied the anilin-oil-gentian-violet for one and a half minutes, and the Gram solution for one and a half minutes instead of the one- and two-minute periods of the committee.

In all cases we made the stain on young 20° agar cultures (not over five days old), and in each case the test was made in duplicate at different times. When the results of the two tests coincided, the culture was recorded as positive or negative. Cultures which gave one positive and one negative test, or in which the stained and decolorized appeared in about equal proportions, are recorded in an intermediate class.

Flagella.—As a result of the work of Ellis (1902), we have devoted considerable time to the study of motility among the cocci. This author reported the finding of spores and flagella in various streptococci and sarcinæ, and Arthur Meyer carried this position to an extreme in the statement that "the researches of Ellis have rendered it doubtful whether there are any species of bacteria which entirely lack flagella" (Meyer, 1903). We examined a number of cultures very carefully, transferring them at frequent intervals on different media, according to the general plan adopted by Ellis. We found in almost every case active vibratory movements, with a tendency to incomplete rotation, the successive jerks sometimes producing a gradual translation across the field. This type of behavior is entirely different from the true motility characterized by slow, steady revolution, which appears in such forms as *S. agilis*. We are convinced that most of the cocci are non-motile, while a few forms show true movement; it is with this type of motility that clearly stainable flagella have been found associated. The study of this character is

therefore of significance. It is questionable, however, whether it is one of the most important characters in this group of bacteria. It appears from the published descriptions of species that this property is not correlated with any other character, arising independently in forms exactly resembling non-motile forms in every other respect. On account of its rarity and this apparent lack of correlation with other differences, as well as on account of the difficulty of studying it, the property of motility has been so far omitted from the present study.

Spores.—The experiments carried out by Ellis (1902) strongly suggest the presence of specially resistant cells in old cultures of the cocci. His figures are, however, by no means conclusive as to the existence of true spores. In the absence of any observations as to germination, we have not felt that the evidence warranted extensive microscopic study of this character.

Fission.—A study of the conditions influencing the growth-forms of the Coccaceæ should be of considerable interest. Pairs and chains are apparently associated with meager, and groups and packets with more abundant, development. The effect of the general rate of growth must, however, be modified by the rate at which cell-wall and cell-protoplasm, respectively, are formed.

A careful study of the method by which these groupings arise in cell-division, such as could be made by the use of Hill's hanging-block method, would no doubt throw much light on all such points, and should precede any final conclusions as to the relationships of the cocci. In examining a large number of organisms, however, the agar block would have proved too time-consuming. We have therefore limited ourselves to the observations made on stained preparations from ordinary cultures.

Capsules.—Considerable preliminary work failed to indicate any constant differences in capsule formation among the cocci studied. This character appears to be of considerable value among the diplococci (Buerger, 1904); but even with them it varies markedly with the medium used for cultivation. We cultivated certain selected organisms in broth at 20° and at 37°, on nutrient gelatin, acid gelatin, alkaline gelatin, anaërobic gelatin, and Nährstoff-Heyden agar, and examined them at intervals by Welch's staining method. In every case capsules were apparent at some stages, being most

strongly developed in old cultures and on poor media like the Nährstoff agar. This character has not seemed to us of sufficient diagnostic value to be included in our routine examinations.

Involution and degeneration forms.—In numerous examinations of old cultures we found no involution forms of special significance. As noted above, swollen and oval forms are more apt to occur in old cultures of cocci, but they are not sufficiently definite to warrant record.

4. CULTURAL CHARACTERS.

In a study of this sort we have necessarily included only those tests which reveal definite and independent variable characters. Most of the commonly observed cultural characteristics are the secondary results of a few fundamental properties which can be observed on one medium as well as on several. For this reason we have eliminated a number of the ordinary media from our routine. The general character of the growth is approximately the same on *agar*, *blood serum*, *potato or Nährstoff*, except that agar has always markedly more growth and potato often none. An organism producing abundant chromogenic growth on agar will give good growth and some pigment on the other media. The streptococcus growth on agar gives restricted and veil-like growth on serum and Nährstoff, and usually no growth on potato. In other words, Nährstoff agar, serum, and potato are simply poorer media than agar, and show no specific characteristics other than those due to feebleness of growth. Blood serum may be useful in other groups to show a special type of liquefaction, but in a preliminary study of 50 of our cultures we never found this to occur, and it is nowhere recorded in published descriptions of the Coccaceæ. In 25 out of 50 cultures grown on potato no growth occurred, and in no case have we observed discoloration. These media have therefore been omitted. This action is in accordance with the conclusions of the Committee on Standard Methods (1905), in considering their value for general diagnostic use.

Nutrient broth.—In the group of the cocci we have not found that any information of definite value could be derived from a study of broth cultures. None of the forms studied form a surface pellicle or produce any characteristic odor. There remain to be observed only two features—turbidity and sediment—which in our

judgment depend directly on other properties, such as the general vigor of growth and the size of the cell aggregates. Both turbidity and sediment vary markedly with the age of the culture; what is first turbidity later settles to form sediment, as the waste products of the bacteria check their development. The amount of either depends on the activity of growth. A constant difference often appears between cultures which early in the course of development show considerable turbidity with little or no sediment, and those which almost at once develop a heavy sediment with colony-like masses

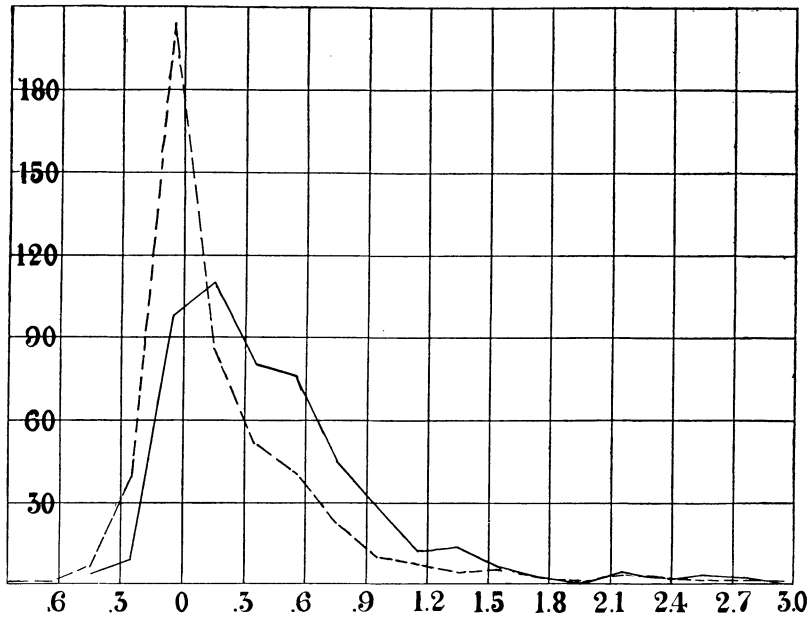

FIG. 2.—Acid production of 500 cocci; in dextrose broth =————, and lactose broth = - - - - - -. Abscissæ, acidity in per cent normal. Ordinates, number of cultures.

of growth clinging to the walls of the tube. This difference, however, appears to be correlated with the growth-form and general vigor of the coccus. Organisms of the Streptococcus type with cells separating readily, which show faint surface growth, produce chiefly turbidity; while organisms like Sarcina with large cell aggregates and rich surface growths, show heavy sediment.

Gelatin plates.—Minute differences in the macroscopic and microscopic appearance of colonies on gelatin are given great

weight in German systems of classification. Certain special characteristics do, indeed, appear in old gelatin colonies of the cocci after several weeks of incubation. Colonies may remain almost spherical; or they may expand in flat, disclike growths with terraced edges. Sometimes a distinct boss appears at the center, surrounded by a flatter area. The edges may be entire, or more or less deeply scalloped, and the edges of the scallops may be produced inward in folds. Concentric rings sometimes appear in the interior of the colony, or zones of partially liquefied gelatin around its periphery. Some of these characters vary without any apparent reason, as different colonies on a plate show different characteristics; this is perhaps due to differences in the position of the original cell relative to the gelatin surface. Most of them are profoundly modified by variations in the amount of moisture in the gelatin and in the atmosphere above. In a series of comparative studies with different conditions of incubation we found that highly characteristic colonies of granular structure, with deeply lobed edges and indented surfaces, could be produced by cultivation in an incubator whose atmosphere was kept dry by calcium chloride. Dunham (1903) has pointed out the wide differences which may be due to slight variations in the physical properties of the gelatin used. Those differences which are really characteristic of the organisms themselves appear to be related to two fundamental powers: the general vigor of growth and the liquefying power. It may be possible that other differences exist in old gelatin colonies which are really characteristic, but in the present state of knowledge it seemed best to omit the gelatin plate in favor of more definite tests. Liquefying power and general vigor of growth are observed in the gelatin stab and the agar streak respectively.

Gelatin tubes.—All our cultures have been studied in the gelatin tube, but only the single character of the amount of liquefaction has been systematically recorded. The distinction between different non-liquefying colonies lies in the amount of surface growth and the color, both of which characters are more easily studied on the agar streak. The character of the surface growth, like that of the gelatin plate colony, does not appear in this group to offer any character of diagnostic value, and all the cocci grow fairly well in the stab.

Among the liquefying forms we have not found the shape of the liquefaction of sufficient constancy to be recorded. Whipple (1902) has strikingly shown the uncertainty of this character—almost every possible type appearing in media made with slightly different commercial gelatins. The Committee on Standard Methods (1905) has also omitted this property.

The amount of liquefaction of gelatin was therefore the only character recorded on the gelatin stab. The method by which this was measured will be described under "Biochemical Reactions."

Agar plates.—The same reasons which led us to omit the gelatin plate militate against the use of the agar plate as a diagnostic test. Constant differences which exist between colonies are slight and depend on a few fundamental properties which may be more easily observed on other media, notably on the agar streak.

Agar tubes.—The general conclusion from what has been said in this discussion of cultural characteristics is that in the cocci a single medium is sufficient for their determination. We should, however, deprecate any extension of this conclusion to other groups where the gelatin stab or the plate culture may yield information of definite value. Even among the cocci further study may show constant and characteristic differences in gelatin colonies, and if this should be the case, no one could fail to welcome an addition to the meager list of diagnostic characters at our disposal. In the absence of evidence as to the value of these media, we feel it unwise to repeat tests mechanically and without any definite purpose, merely because they have had an important place in the historical development of the science.

All cultural characteristics have therefore been observed in the agar tube. A combined streak and stab was made on a slant surface, and the cultures were uniformly studied after incubation for two weeks at 20° C. Cultures of different age exhibit marked differences, but the characters of the old cultures are the outcome of those of the new. Comparative studies with lactose agar and glycerin agar showed neither to be as favorable a medium as ordinary nutrient agar.

In order to obtain a comparative idea of cultural characters we examined two weeks' agar streaks of the whole 500 cultures

at the same time. We are somewhat surprised to find that the visible differences between the cultures were due almost wholly to two properties—chromogenesis and the general vigor of surface growth. There was a distinction in luster between a large majority of the

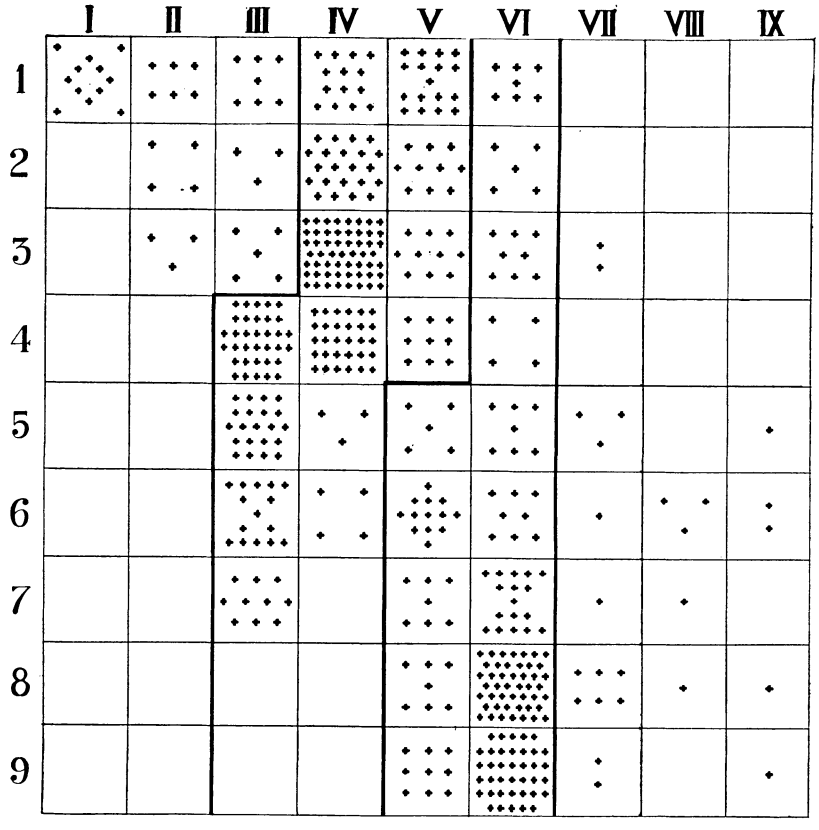

FIG. 3.—Distribution of 500 cocci according to chromogenesis. Roman numerals, hues; Arabic numerals, chromos.

cultures which had smooth and shining surfaces, and a few which were dull and rough. This difference appears, however, to be due simply to the relative amount of growth and moisture in the tube. Faint growths are moist and shining, while heavy growths in tubes which do not contain much moisture show the dry, rough, dull appearance. The "white chromogens" showed another slight difference, varying from an opaque porcelain white to a duller and

more translucent growth of indefinite color and somewhat shiny appearance; but there was no sharp boundary to separate the types. For the present we have omitted this character, although it may prove to be of importance in more detailed work.

We have therefore noted, as cultural characters on the agar streak, only the color production and the vigor of surface growth. The method of studying the former character will be described under "Chromogenesis." Under "Vigor of Surface Growth" we found it possible to distinguish five different types. Grade 1 includes forms of the Streptococcus type which form only a very faint, veil-like, growth, or a few translucent dotted colonies on the surface. Grade 2 is reserved for a somewhat more abundant, but still meager, growth. Grade 3 corresponds to a good, but not abundant, streak; Grade 4, to an abundant growth; and Grade 5, to a very heavy surface development.

The relation to free oxygen is distinctly involved in the vigor of surface growth, and the agar streak also served for the study of various other biochemical reactions. Inhibition of growth by acidity and alkalinity of media, temperature relations, and pigment formation were all recorded on this medium under conditions to be described below.

5. Biochemical Reactions.

Action upon milk.—Milk is a favorable nutrient medium for bacterial growth because of its rich food properties, and in many groups it gives important information, but it has no specific diagnostic value for the Coccaceæ, as all the changes it undergoes are correlated with those which occur in sugar broths and with the general activity of the organism. No coagulating enzymes and casein-digesting enzymes are found in this group, so far as we are aware, and no gas or odor is produced. The only changes which the cocci effect in milk are therefore the production of acid or alkali, coagulation and decolorization of the litmus.

Decolorization has no significance, except that it indicates the general activity of the organism. When the organism is most active, it uses up the oxygen and reduces the litmus, which is accordingly decolorized, and, conversely, when activity grows less, oxygen diffuses from the surface making the litmus pink again.

Coagulation depends upon the amount of acid produced, and is more easily studied in sugar-broth cultures.

Action upon carbohydrates.—The characteristics usually observed in sugar broth are turbidity and sediment, relation to oxygen, gas production, and acid production. We have given reasons, in discussing nutrient broth, for considering turbidity and sediment unimportant, and the relation to oxygen is most sharply defined by surface growth in the agar tube. None of the cocci, so far as known, produce gas, and there remains only acid production to be recorded. For this purpose ordinary straight tubes were used. The sugars tested were dextrose and lactose. Saccharose has been omitted for the present, for lack of time. A preliminary test indicated that this sugar is less commonly fermented by the cocci than are dextrose and lactose.

The media were made up in the usual manner with 2 per cent of the sugar to be tested. The reaction was made about neutral, and after tubing and sterilization it was usually between 0.5 and 1.0 per cent. After standing for two weeks sterile blanks showed a slight further rise in acidity, so control tubes were always kept with each batch inoculated and titrated at the end of the experiment. After considerable preliminary experimentation, it was decided to titrate with phenolphthalein as an indicator in the cold. Methyl orange is not sensitive to the organic acids and gives a poor end-point. With phenolphthalein a comparative series of titrations made on the same tubes, first cold and then boiling, showed slightly higher results by the latter method. Evidently heating increases the apparent acidity more by the breaking-up of unstable compounds than it decreases it by driving off carbon dioxide. The cold method was therefore used. To 5 c.c. of the sugar broth, grown for two weeks at 20°, was added 95° c.c. of distilled water and two or three drops of phenolphthalein. This was titrated against $\frac{N}{20}$ NaOH and from the value obtained was subtracted the acidity of the blank controls titrated at the same time. All tests were made in duplicate, and the final value recorded as the acid or alkali production of the organism is the difference between the average of two titrations of tubes in which it had grown for two weeks and

the average of two blank controls. No determination was made of the rate of acid production as distinguished from this total final acidity, though such observations might be of much interest.

Action upon nitrates.—Data with regard to the reduction of nitrates by the cocci are extremely meager, the presence or absence of this character being recorded in very few of the published descriptions. It seems, however, to have a fair degree of definiteness, and we have included it as a qualitative test in our routine. Each organism was inoculated into a series of 10 tubes of standard nitrate solution. After seven days' growth at 20° the tubes were tested for nitrites and ammonia by the regular method prescribed by the Committee on Standard Methods (1905). The test for nitrates was omitted after it was found that all the cultures, out of a considerable series tested, gave positive results, without exception. The results of the tests for nitrites and ammonia are expressed in the number of tubes which gave positive results, out of the 10 which were tested. In view of the fair constancy of the reaction as observed, we regret that this test was not made quantitative.

Production of indol.—A preliminary examination of some 50 cultures showed no production of indol in any case, and a study of the literature of the cocci indicates that this property is very rare, if it ever occurs, in this group. It was therefore omitted from our routine.

Inhibition of growth by acidity and alkalinity of media.—This character is of considerable importance and warrants careful study, but it is obviously a difficult property to observe in a large series of cultures, and we have not attempted to use it in the present investigation. A preliminary examination of 33 cultures, the results of which are shown in the table, indicated that 1 per cent is the optimum acidity for a majority of these organisms, and that an excess of acidity over this amount is more generally fatal than an alkaline medium.

OPTIMUM REACTION FOR GROWTH AND COLOR PRODUCTION.

NUMBER OF ORGANISMS.

Optimum Reaction	−1.0	−.5	0	+.5	+1.0	+1.5	+2.0
Growth	2	4	5	6	9	6	1
Color	3	1	0	3	9	6	3

Relation to free oxygen.—The Committee on Standard Methods (1905) recommends that the relation of bacteria to oxygen be studied by the comparison of cultures made under normal, and under anaërobic, conditions. A preliminary study of 50 cultures made in this way led to the belief that such a procedure is unnecessary among the cocci. All but two of the cultures studied showed some growth under anaërobic conditions, but the growth was in most cases meager. It became evident that there are two main types of organisms: those which, like Streptococcus, grow only feebly on the surface of aërobic agar, and which grow equally well under anaërobic conditions; and those, like Sarcina, which form abundant surface growths under aërobic conditions, and under anaërobic conditions grow feebly like Streptococci. In other words, there is little difference between the anaërobic cultures of the cocci. Therefore, for purposes of classification we have considered the study of the aërobic surface growth a sufficient measure of the relation to free oxygen, as well as of general vigor. The five grades recorded under vigor of surface growth correspond fairly well to four grades of aërobiosis, from forms anaërobic and facultatively aërobic, to forms which are strong aërobes.

Temperature relations.—There are two points of special importance which ought to be determined in studying temperature relations, the optimum temperature and the high death-point. The death-point at extremely low temperatures is too indefinite to be attempted, and the extreme limits of growth, although desirable data may be omitted as less important than the other two properties.

For the determination of the optimum temperature we first made a series of preliminary studies by comparing agar cultures grown at 10°, 20°, 37°, 45°, and 56°. We found two cultures growing better at 20°, 18 developed equally well at 20° and 37°, 22 showed an optimum at 37°, two grew equally well at 37° and 45°, and four grew best at 45°. These conclusions refer only to the amount of growth, color production being in most cases most active at 20°. From these results we concluded that the information to be gained by cultures grown below 20° and above 37° would be scarcely commensurate with the labor involved, and we have limited our observation to the comparison of growth and color production at 20° and 37°.

Cultures were grown for this purpose on agar at 20° for two weeks and compared by inspection. Amount of growth and depth of color were recorded in five arbitrary grades as follows: growth or color production, much better at 20°, somewhat better at 20°, equal at the two temperatures, somewhat better at 37°, and much better at 37°.

Thermal death-points were included in the original plan of our experiments and have now been made on 87 cultures. The process used is to inoculate from three- to five-day-old agar cultures into broth tubes brought to the desired temperature in a water-bath heated electrically by a platinum coil, and to expose them for 10 minutes. The tubes are then cooled and incubated at 37° for six days. At the end of that time, streaks are inoculated from the broth tubes in order to make sure by characteristic growth that the organisms orginally inoculated are present. Tests are made from 55° up to the point where growth fails. The process is so tedious that we have been unable to complete the work, and must omit this property for the present. The general results so far obtained are as follows:

THERMAL DEATH-POINTS.

NUMBER OF CULTURES KILLED AT VARIOUS TEMPERATURES.

Temperature	50°	55°	60°	65°	70°	75°	80°
Cultures	2	5	24	17	16	22	1

Pigment formation.—The production of color by the bacteria is not only markedly affected by contemporaneous conditions of cultivation, but may be profoundly modified by selective action or by the effect of antecedent environment. First, of the conditions which temporarily affect the production of color, without modifying the inherent chromogenic power of the organisms, may be mentioned the medium, the presence of free oxygen, and the temperature. In some bacteria, media of low nutritive value, like potato and Nährstoff, appear to favor pigment formation, but with the cocci this is not generally the case. Agar has, on the whole, shown a better development of chromogenesis than any other medium tested. The presence of free oxygen is generally an essential for color production, stab growths being almost invariably lightly colored. We have found a single exception to this rule in a coccus which produces

an orange pigment of much deeper tint in the stab than on the surface. In comparing color at different temperatures we have found, in general, a much better pigment formation at 20° than at 37°. A deep orange growth at the lower temperature may often correspond to a white one at 20°. This effect has been recorded in our routine studies, and will be more fully discussed later with their results as a basis. Besides these temporary modifications of the chromogenic power, the actual color of cultures may be indirectly affected by certain other factors. The general vigor of growth is naturally correlated with apparent depth of color, and the dryness of the atmosphere increases its intensity by evaporating moisture and concentrating the pigment. Both these factors, increase in the total amount of pigment and concentration by evaporation, produce a progressive deepening of color in old cultures.

Even if the temporary conditions of cultivation are quite constant, the chromogenic power of an organism may be modified by its previous history. In thermal death-point observations we have found interesting cases of this sort. Some streaks made from broth cultures which had been exposed to a temperature of 50° or 55° were deeper in color than was the normal for the organism, but in most cases they were much lighter. Sometimes streaks made from a yellow or an orange chromogen after such treatment were almost colorless, although successive transfers generally restored the normal properties. Finally, we have noticed in our work spontaneous variations in chromogenesis such as have been recorded by Neumann (1897), Conn (1900), and Sullivan (1905). The latter authors note that on a plate sown from a single colony there may develop colonies varying appreciably in shade from which selections of the extremes will produce quite distinct types. Neumann records the sudden appearance of widely different strains, as sectors in old and carefully sealed stab cultures. We have observed both phenomena in our cultures, and are inclined to attribute the first, and, more doubtfully, the second, to variation rather than contamination.

In spite of all these facts it is clear that, as the cocci normally occur in nature, chromogenesis is one of their most distinct and significant differences. In any series of plates sown with washings from the skin four well-marked types—red, yellow, orange, and

white—are pretty certain to occur. We have therefore included chromogenesis as one of our routine tests. The variations due to past and present environment are, of course, easily excluded by the maintenance of constant conditions. Our stock cultures were in all cases kept on agar at 20°, and cultures for chromogenesis were grown on that medium, and at that temperature, for two weeks. In order to avoid the apparent differences due to the vigor of growth or to evaporation, a portion of the growth was removed on a loop needle and spread out on white drawing-paper with a rough surface. After drying at the room temperature, the color was compared with an arbitrary standard scheme.

The color chart used for matching these colors was devised after a very careful study of the colors actually found among the Coccaceæ, and includes nine hues ranging from white through lemon-yellow, light cadmium, medium cadmium, lemon-yellow and cadmium orange, red and cadmium orange, to two different combinations of red with lemon-yellow. We have used under each hue, nine different chromas, obtained by successively increasing washes of the hues on white paper. The hue in each case is recorded by a Roman numeral; the chroma, or number of wash, by an Arabic subscript.

Liquefaction of gelatin.—The liquefaction of gelatin, like the property of pigment production, has been shown to be subject to fluctuating variations. Conn (1900) was able by selection to obtain from a single culture of a milk coccus a rapidly liquefying form, and one with almost no peptonizing power. Smith (1900) records similar experiences with colon bacilli and forms of *B. proteus*. There appears to be little correlation between liquefaction and any other power, since it is so common in widely separated groups of bacteria to find organisms differing in this respect, while identical in all other properties.

In studying liquefaction we have determined only the amount of the action exerted by each organism. The shape of the liquefaction in the stab culture has been shown by Whipple (1902) to vary within the widest limits, with slight differences in the character of the medium, and the Committee on Standard Methods (1905) has omitted this character from its list.

For determining the amount of liquefaction we have used the

method suggested by Clark and Gage (1905), which consists in inoculating gelatin tubes of 10 mm. diameter by spreading a suspension of the culture over the surface. Liquefaction proceeds in a stratiform fashion, and its amount may be read off in centimeters. With such a method one may determine the rapidity of liquefaction either

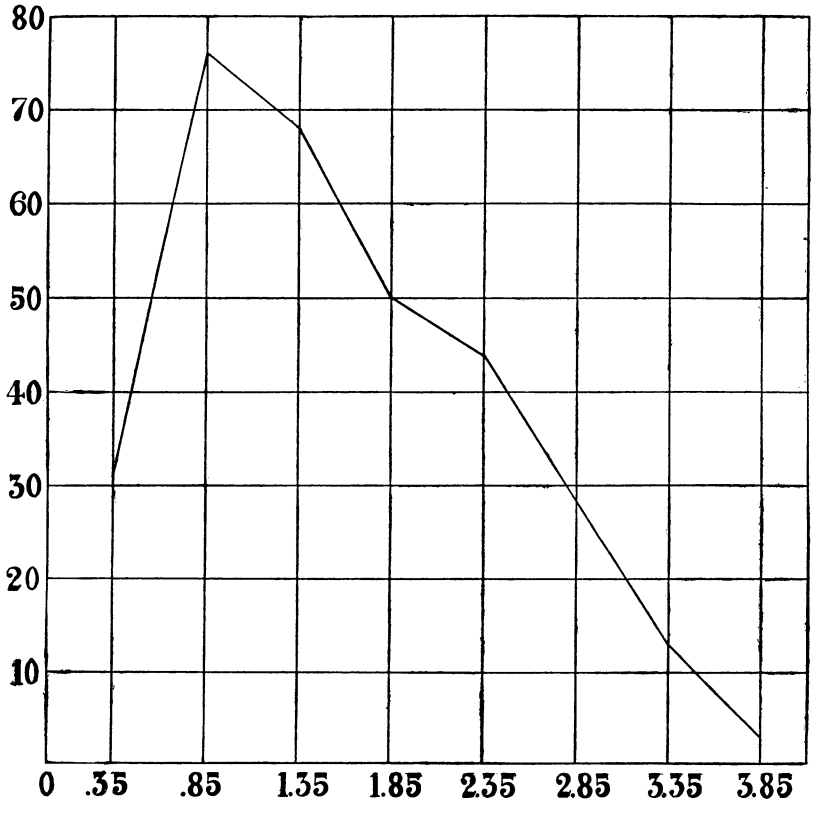

FIG. 4.—Liquefaction of gelatin by 314 cocci. Abscissæ, liquefaction in cm. Ordinates, number of cultures.

by the number of days required to reach a fixed point, or the final amount of liquefaction. In general, these two values are pretty closely correlated, but in a preliminary study we found that the final differences are somewhat sharper as well as easier to record. We have therefore adopted as our routine measure of liquefying power the depth of liquefaction after 30 days.

Supplementary tests.—Many other tests than those mentioned are sometimes used in bacterial diagnosis, but none have seemed suited to the present study. The questions of pathogenicity and agglutinative power are so shrouded in confusion as to be unpromising. Meyer (1902) considered serum reactions of diagnostic value among the streptococci, and Kolle and Otto (1902), and Otto (1903), obtained good results with the staphylococci. On the other hand, Aronson (1903), Fischer (1904), and Kerner (1905), after very thorough investigations, came to the conclusion that these properties among the streptococci are so erratic as to be quite valueless in systematic work. From a general survey of the literature of the group, it seems probable that the properties connected with infection and immunity are likely to be too easily modified to prove helpful in classification.

The test for liquefaction of starch is one which it seems logical to include with those which show the relation of an organism to gelatin and the sugars; and we made some experiments with the starch media introduced by Smith (1905). It appeared that certain cocci did exert an amylolytic action and the study of this character would probably prove of considerable interest. It has been omitted for the present, for lack of time.

III. RESULTS OF THE INVESTIGATION.

The characters observed and the terms in which they are recorded may be summarized as follows:

1. *Habitat.*—Recorded as 1 (diseased conditions); 2 (normal body); 3 (water); 4 (earth); or 5 (air). The significance of these various habitats has been more fully discussed above. It should be noted that Group 5 includes certain laboratory cultures whose origin was unknown.

2. *Grouping of cells and dimensions.*—Observed in stained preparations, made from 20° agar cultures less than five days old. Grouping recorded as 1 (packets present); or 2 (packets not present). Extreme dimensions recorded in micromillimeters to the nearest 10th.

3. *Relation to Gram stain.*—Observed on two occasions on 20° agar cultures less than five days old. Treated with anilin-oil-gentian-

violet for one and one-half minutes; Gram's solution, one and one-half minutes; 95 per cent alcohol, three minutes. Counterstained with Bismarck brown for one-half minute. Reaction recorded as − (decolorized in both tests); ± (stained once and decolorized once); or + (stained in both tests).

4. *Vigor of surface growth on agar streak after 14 days at 20°.*—Recorded as 1 (very faint); 2 (meager); 3 (good); 4 (abundant); or 5 (very heavy).

5. *Amount of acid produced in 2 per cent dextrose broth after 14 days at 20°.*—Determined by titration against $\frac{N}{20}$ NaCl in the cold with phenolphthalein as an indicator. Recorded value is the difference between inoculated tubes and sterile controls, expressed in cubic centimeters to nearest 10th.

6. *Amount of acid produced in 2 per cent lactose broth.*—Same conditions as under 5.

7. *Formation of nitrites in nitrate solution.*—Recorded value is the number of tubes giving positive test for nitrites out of a series of 10, grown for seven days at 20°.

8. *Formation of free ammonia in nitrate solution.*—Same method as under 7.

9. *Comparative growth after 14 days' growth on agar streak at 20° and 57°, respectively.*—Recorded as 1 (much more vigorous at 20°); 2 (more vigorous at 20°); 3 (equal); 4 (more vigorous at 37°); or 5 (much more vigorous at 37°).

10. *Chromogenesis.*—Hue and chroma of pigment produced on agar at 20° after 14 days, determined by comparison with color scheme as described later.

11. Depth in cm. of gelatin liquefaction in tube of 1 mm. diameter after 14 days at 20°.

It would be well to extend this series of tests by a study of the cell-grouping in broth, motility, fission on the agar block, fermentation of saccharose, effect of acid and alkaline media, and the thermal death-point.

1. HABITAT.

The distribution of the cultures isolated among the various habitats was as follows: (1) diseased conditions, 59; (2) normal body,

170; (3) water, 95; (4) earth, 67; (5) air, 109. It is probable that this deviates from a representative sampling of the cocci in nature by laying too great stress on the saprophytic forms. It is difficult to find cocci at all in earth and water, whereas they are present on the surfaces of the body in enormous numbers. The majority of this group appear to be parasitic or semiparasitic in habit. At the same time, the fairly equal weight given to the saprophytic forms helps to bring out the differences between the two main groups, those living in or on the animal body (1 and 2), and those living in the outer world (3, 4, and many of 5).

We have prepared tables to show the distribution of each character among various habitats, and the relations shown are so suggestive as to warrant rather full discussion. In Table 1 is given the correlation between habitat and cell-grouping, and it is at once evident that the sarcinæ occur in greater proportion outside than inside the body.

In this and succeeding tables the figures represent the number of cultures showing each combination of characters out of the 500 studied.

TABLE 1.

CORRELATION BETWEEN HABITAT AND CELL-GROUPING.

	Diseased Conditions	Normal Body	Water	Earth	Air
No. packets	44	145	45	33	78
Packets	15	25	50	34	31

Whereas packets are more abundant in earth and water, the other forms—chains, plates, and irregular groups without sarcinæ—make up two-thirds or more of the parasitic forms.

TABLE 2.

CORRELATION BETWEEN HABITAT AND GRAM STAIN.

Gram Stain	Diseased Conditions	Normal Body	Water	Earth	Air
−	14	12	46	37	36
±	17	50	29	18	32
+	28	108	20	12	41

The distribution of our cultures according to their relation to the Gram stain brings out a similar condition. The cultures giving

a consistent positive reaction make up far more than half the total among the parasitic forms from the first two habitats, and less than one-fourth of the forms from water and earth. The air cultures in almost all cases exhibit an intermediate relation, as would be expected, since they must contain forms from both sources. A positive reaction to the Gram stain is evidently closely correlated with life in and on the animal body.

TABLE 3.

CORRELATION BETWEEN HABITAT AND SURFACE GROWTH.

Surface Growth	Diseased Conditions	Normal Body	Water	Earth	Air
Very faint	0	16	3	0	0
Meager	1	15	2	2	1
Good	23	98	38	24	32
Abundant	27	34	38	31	60
Very heavy	8	7	14	10	16

The abundance of surface growth also varies with the habitat. Very faint and meager growths are fairly abundant in the forms from the surfaces of the body, as would be expected, since our culture media are unfavorable for the more strictly parasitic forms. On the other hand, a majority of the earth and water cocci show abun dant or very heavy surface growths. The air forms are characterized by particularly abundant development, as would naturally be expected, since only the vigorous cells probably survive drying and dispersal through the air.

TABLE 4.

CORRELATION BETWEEN HABITAT AND THE FERMENTATION OF DEXTROSE BROTH.

Acid Production per Cent of Normal	Diseased Conditions	Normal Body	Water	Earth	Air
0.0 and alkaline	12	12	39	28	20
0.1–0.2	7	33	22	24	24
0.3–0.6	20	82	19	10	25
0.7–2.0	17	38	12	5	34
2.0 and over	3	5	3	0	6

TABLE 5.

CORRELATION BETWEEN HABITAT AND THE FERMENTATION OF LACTOSE BROTH.

Acid Production per Cent of Normal	Diseased Conditions	Normal Body	Water	Earth	Air
−0.2 and more alkaline	6	12	9	10	13
−0.1 and 0.0	23	37	62	42	40
0.1–0.4	15	64	16	11	32
0.5–1.4	11	49	5	4	19
1.5 and over	4	8	3	0	5

In examining Tables 4 and 5, which show the fermentative power of dextrose and lactose broth, the fundamental difference between the parasitic and saprophytic cocci is again made evident. In the first two groups, from the animal body, over two-thirds of the cultures produce more than 0.3 per cent of normal acid; while among the earth and water forms two-thirds of the organisms form less than this amount. With lactose the same law holds. Two-thirds of the cocci from the normal body produce acid in lactose, against less than one-third of the water and earth forms. The air cultures show an intermediate relation.

TABLE 6.

CORRELATION BETWEEN HABITAT AND REDUCTION OF NITRATES.

	Diseased Conditions	Normal Body	Water	Earth	Air
No reduction	44	147	75	44	72
Nitrites formed	10	19	13	10	18
Ammonia formed	7	7	8	13	30

The property of nitrate reduction does not appear to be related to habitat in any such direct way as the other characters studied. The air cocci, however, show a peculiarity of considerable interest, nitrite formation being common, and ammonia formation very common, among them.

TABLE 7.

CORRELATION BETWEEN HABITAT AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum	Diseased Conditions	Normal Body	Water	Earth	Air
20°	9	11	29	23	11
20° or 37°	36	112	56	42	89
37°	14	47	10	2	9

While a majority of the cultures studied grow indifferently at 20° or 37°, it appears from Table 7 that among the parasitic forms a fair proportion are favored by the body temperature, while more of the earth and water forms develop best at 20°. With regard to the optimum temperature for color formation, no definite relation with habitat appears, except as involved in the double relation between chromogenesis and habitat, and chromogenesis and the optimum temperature for color formation. These figures are therefore omitted.

TABLE 8.

CORRELATION BETWEEN HABITAT AND CHROMOGENISIS.

Chromogenesis	Diseased Conditions	Normal Body	Water	Earth	Air
White	4	17	5	1	13
Yellow	33	37	76	50	58
Orange	21	116	6	10	28
Red	1	0	8	6	10

Table 8 brings out some of the most definite relations yet considered, between habitat and chromogenesis. It is evident that the white and orange forms are largely parasitic, and the yellow and red forms as distinctively saprophytic. More than half of the white and more than two-thirds of the orange chromogens come from the first two habitats, while only one-third of the yellow forms have such an origin. The distribution of the red pigment-formers is even more notably saprophytic. Only one culture out of 25 occurred among the 229 cultures from the body.

TABLE 9.

CORRELATION BETWEEN HABITAT AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (Depth in cm.)	Diseased Conditions	Normal Body	Water	Earth	Air
0.0	13	68	43	26	36
0.1–1.5	24	36	42	30	45
1.6 and over	22	66	10	11	28

The table for habitat and gelatin liquefaction (Table 9) shows this property occurring among the earth and water forms to a less degree than among the parasitic cocci. This fact, and the fact that the parasitic forms are high acid-producers, as noted above, are of practical significance in connection with the bacteriological analysis of water. It has long been suspected that acid production and gelatin liquefaction were associated with intestinal organisms, and we have here a measure of the truth of this proposition, in the case of the cocci at least. From the food conditions which obtain in the alimentary tract, and to a less extent on the outer surfaces of the body, it is natural that these properties should be highly developed.

The forms from Habitat II (the surfaces of the normal body) group themselves most abundantly at two extremes, 68 being non-

liquefiers and 66 active liquefiers, while only 36 occupy the intermediate position, which is of most frequent occurrence in all the other habitats. It is probable that this may be accounted for by the presence, in Habitat II, of two distinct series—the white and colorless forms, which, as we shall see later, are non-liquefiers, and the orange forms, which peptonize strongly.

From a general survey of our habitat studies it is evident that the forms from the body exhibit quite different characteristics from those of the water and earth cocci. The parasitic forms generally react positively to the Gram stain, give only fair surface growths on the surface of artificial media, produce acid in dextrose and lactose, grow best at 37°, produce no pigment or a white or an orange pigment, and liquefy gelatin. The saprophytes, on the other hand, are more apt to occur in packets, to be Gram-negative, to grow abundantly on artificial media, best at 20°, to produce yellow and red pigments, and to exert little action on sugars and gelatin. The air cocci are generally intermediate in character between the two groups, but show special powers of nitrate reduction.

2. GROUPING OF CELLS, AND DIMENSIONS.

The cocci, as noted above, were divided into two classes only, according to the character of the cell aggregates; 155 cultures showed the packets or sarcina-grouping, and 345 did not.

TABLE 10.

CORRELATION BETWEEN CELL-GROUPING AND GRAM STAIN.

Gram Stain	Irregular Groups and Chains	Packets
−	75	70
±	98	48
+	172	37

We have pointed out above that packets are most abundant among the saprophytic cocci of the earth and water. Table 10 shows the relation between cell-grouping and the Gram stain, clearly indicating that the packets tend to be Gram-negative, while a majority of the other forms give a positive reaction.

Table 11 shows a distinct correlation between cell-grouping and the vigor of surface growth. A large majority of the non-packet-

forming organisms produce only very faint, meager, or good growths while a large majority of the sarcinæ form abundant or very heavy

TABLE 11.

CORRELATION BETWEEN CELL-GROUPING AND SURFACE GROWTH.

Surface Growth	Irregular Groups and Chains	Packets
Very faint	18	1
Meager	16	5
Good	169	46
Abundant	132	58
Very heavy	10	45

growths. The fact that the packet-forms flourish on artificial media should naturally result from their saprophytic origin.

TABLE 12.

CORRELATION BETWEEN CELL-GROUPING AND THE FERMENTATION OF DEXTROSE BROTH.

Acid Production (Per Cent Normal)	Irregular Groups and Chains	Packets
0 and under	58	53
0.1–0.2	56	54
0.3–0.6	128	27
0.7–2.0	87	20
2.0 and over	16	1

TABLE 13.

CORRELATION BETWEEN CELL-GROUPING AND THE FERMENTATION OF LACTOSE BROTH.

Acid Production (Per Cent Normal)	Irregular Groups and Chains	Packets
—0.2 and more alkaline	38	11
0.1 and 0.0	113	91
0.1–0.4	104	35
0.5–1.4	72	16
1.5 and over	18	2

A marked correlation between the packet-forming organisms (presumably saprophytic) and the fermentation of sugars is manifested in Tables 12 and 13. Taking Table 12 as an illustration, there will be found to be about 60 per cent of the packet-formers producing alkali, or, at most, fermenting dextrose but slightly (to 0.2)—almost none of the organisms occurring in the class of highest acid producers. Conversely, 70 per cent of the organisms which do not form packets produce acid from 0.3 up to the highest amount.

The power to reduce nitrates appears with about equal regularity in both our morphological groups.

TABLE 14.

CORRELATION BETWEEN CELL-GROUPING AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	Irregular Groups and Chains	Packets
20°	40	43
20° or 37°	237	98
37°	68	14

A slight but distinct correlation appears between grouping and optimum temperature for growth. In each case most of the cultures grow at 20° or 37° indifferently; but a fair proportion of the more saprophytic sarcinæ show better development at 20°, while among the other forms more find an optimum at 37° than at 20°. With regard to the optimum temperature for color formation there is a slight difference, only two-fifths of the sarcinæ showing more chromogenesis at 20° than at 37°, against one-half of the other cultures. This, as we shall see later, is probably connected with a difference in chromogenesis.

TABLE 15.

CORRELATION BETWEEN CELL-GROUPING AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (cm.)	Irregular Groups and Chains	Packets
0.0	127	59
0.1–1.5 cm	110	67
1.6 and over	108	29

Table 15, for the relation of gelatin liquefaction to morphology, shows only that the highest grades of liquefaction are somewhat less numerous among the packets than in the other group.

The results obtained with regard to the size of the individual cell were much less suggestive than the facts concerning cell-grouping. Dimensions were measured in all cases on the stained specimens, and were recorded independently on at least two occasions. The attempt was made to note in each case the extreme diameters observed, and the values finally adopted represent the average between the recorded extremes. Individual cells ranged from 0.1 to 2.0 μ. With the packets it was found impossible to determine the maximum size of the single cell, on account of the frequent occurrence of small, recently formed packets which stained as a whole like one cell. We never felt certain that what appeared like a large cell was not

really a group of eight small ones. The sarcinæ in general showed quite small individual units, 0.1 or 0.2 μ as a rule, with no constant deviations. The packets are therefore entirely omitted from the consideration of dimensions.

The average sizes of the 345 cultures, not occurring in packets, are grouped in convenient classes in Table 16 and plotted in Fig. 1.

TABLE 16.

DIMENSIONS OF 345 COCCI.

Size, average, μ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Number of cultures	22	82	113	60	40	12	7	6	1	2

The sizes of the cocci studied are evidently distributed on a fairly normal curve of frequency. The mode is at 0.3 μ and the curve is markedly skew, with infinite extension toward the larger sizes. The important practical point is that the forms measured appear to behave like a fairly homogeneous series varying about a single mode.

We have made tentative calculations of correlation between cell dimensions and other characters, with almost entirely negative result. The only property showing any relation is that of gelatin liquefaction.

TABLE 17.

CORRELATION BETWEEN CELL DIMENSIONS AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (cm.)	Maximum Size 0.3 μ and under	Maximum Size over 0.3 μ
0.0	47	78
0.1–1.5	65	47
1.6 and over	69	39

An appreciable inverse correlation is shown between the size of the cell and the rate of gelatin liquefaction, the smaller cocci liquefying most readily.

3. GRAM STAIN.

We have pointed out above that the reaction of the cocci to the anilin-oil-iodin stain is a variable character, many forms showing a positive reaction on one occasion and a negative reaction when next tested. Nevertheless the test, variable as it is, shows quite constant relations to other characteristics; and we feel convinced that among the cocci where all characters are more or less fluctuating any prop-

erty which on the average shows a definite correlation with other properties has systematic significance.

Of the cocci studied, 145 showed in two tests a Gram negative reaction on both occasions, and 209 two positive reactions, while 146 were once stained and once decolorized. Grouped thus in three divisions, we have seen that the positive reaction is characteristic of the parasitic forms, while saprophytic forms and packets tend to be Gram negative. With surface growth only an insignificant relation appears, the less richly growing forms showing a slightly higher proportion of positive reactions.

TABLE 18.

CORRELATION BETWEEN GRAM STAIN AND THE FERMENTATION OF DEXTROSE BROTH.

Acidity Produced (Per Cent Normal)	Gram Negative	Gram Variable	Gram Positive
0.0 and over.....	49	44	18
0.1–0.2	48	29	33
0.3–0.6	35	45	75
0.7–2.0	12	26	69
2.1 and over	1	2	14

TABLE 19.

CORRELATION BETWEEN GRAM STAIN AND THE FERMENTATION OF LACTOSE BROTH.

Acidity Produced (Per Cent Normal)	Gram Negative	Gram Variable	Gram Positive
−0.2 and more alkaline	20	14	15
−0.1 and 0.0.....	72	82	50
0.1–0.4	34	24	81
0.5–1.4	19	20	49
1.5 and over	0	6	14

The relation between the Gram reaction and the fermentation of carbohydrates is a surprisingly close one. Each line in Tables 18 and 19 showing the distribution of organisms among the grades of acidity forms a regular curve. In each case the mode of the Gram-negative cultures occurs at the neutral point, and that of the Gram-positive cultures at a moderately high acidity, with the doubtful cultures showing an intermediate relation.

TABLE 20.

CORRELATION BETWEEN GRAM STAIN AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	Gram Negative	Gram Variable	Gram Positive
20°	34	29	20
20° or 37°	103	89	143
37°	8	28	46

With nitrate reduction and the optimum temperature for chromogenesis the Gram reaction shows no special relations. With optimum growth temperature, on the other hand, Table, 20 shows a distinct connection. As always, most of the cultures grow equally at both temperatures. Among the decolorized cultures, however, a fair proportion grow best at 20°, while with the positive forms 37° is most favorable. Such a relation would, of course, be expected from the generally saprophytic habit of the negative forms.

The liquefaction of gelatin does not show any distinct relation to the Gram reaction. On the whole, therefore, we may conclude that the cocci which decolorize by Gram are generally earth and water forms, which notably fail to ferment sugars, and which grow best at 20°. The marked correlation with the power of acid production, in the absence of other equally marked relations, seems to invite further study of the physiological basis of these properties.

4. SURFACE GROWTH.

The cocci studied were divided into five groups according to the vigor of surface growth on agar. The first group, of "very faint" growths, includes 19; the second group, of "meager" growths, includes 21 forms; "good" and "abundant" growths occur 215 and 190 times, respectively; and 55 cocci show "very heavy" growths.

TABLE 21.

CORRELATION BETWEEN SURFACE GROWTH AND THE FERMENTATION OF DEXTROSE BROTH.

Acidity Produced (Per Cent Normal)	Very Faint	Meager	Good	Abundant	Very Heavy
0.0 and alkaline	3	2	42	41	23
0.1–0.2	1	3	41	51	14
0.3–0.6	3	11	76	54	12
0.7–2.0	5	4	53	39	5
2.0 and over	7	1	3	5	1

TABLE 22.

CORRELATION BETWEEN SURFACE GROWTH AND THE FERMENTATION OF LACTOSE BROTH.

Acidity Produced (Per Cent Normal)	Very Faint	Meager	Good	Abundant	Very Heavy
−0.2 and more alkaline	2	3	18	22	5
−0.1 and 0.0	2	5	80	87	30
0.1–0.4	5	8	65	49	11
0.5–1.4	2	4	47	27	8
1.5 and over	8	1	5	5	1

We have seen above that the fainter growths are characteristic of the parasitic forms, and the heavier ones of the earth and water cocci. The heavier growths are more common among the packets than with other cell groupings.

In comparing the vigor of surface growth with the fermentation of carbohydrates, a distinct relation appears at the ends of the scale, although the bulk of the growth, under the headings "good" and "abundant," exhibit uniform characteristics. The "very faint" growths, which denote members of the genus Streptococcus, as previously defined, are associated with a maximum of acid production falling in the highest acidity class in each sugar table. On the other hand, the "very heavy" growths are mainly forms which fail to act on either sugar.

TABLE 23.

CORRELATION BETWEEN SURFACE GROWTH AND NITRATE REDUCTION.

	Very Faint	Meager	Good	Abundant	Very Heavy
No reduction	19	18	173	133	39
Nitrites formed	0	2	25	38	5
Ammonia formed	0	1	23	29	12

Surface growth and nitrate reduction show a suggestive relation. Among the very faint growths of the Streptococcus type no reduction occurs, and almost none among the "meager" forms. The "good" and "abundant" groups show an increasing proportion of reducing organisms, and the "very heavy" group shows many ammonia-formers and a fair proportion of nitrite production.

TABLE 24.

CORRELATION BETWEEN SURFACE GROWTH AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (Depth in cm.)	Very Faint	Meager	Good	Abundant	Very Heavy
0.0	19	14	63	80	10
0.1–1.5	0	6	84	62	25
1.6 and over	0	1	68	48	20

With gelatin liquefaction there exists the same group correlation manifest for the other characters. The "very faint" group shows not one liquefier, and the "meager" group very few, while the more vigorous forms exhibit a more even distribution.

In general, our study of surface growth brings out two distinct

groups of organisms. The first group, including the forms with faint or meager surface development, corresponds to the genus *Streptococcus* as defined above. It is sharply characterized by high acid production and the absence of gelatin liquefaction or nitrate reduction. Cocci of this type are characteristically parasitic, and very rarely show the sarcina grouping. On the other hand, the more vigorous forms are generally saprophytic, and frequently show packets. They ferment sugar slightly or not at all, and often reduce nitrates and liquefy gelatin.

5. Fermentation of Carbohydrates.

In measuring the amount of acid produced in dextrose and lactose broth, two check determinations were made in each case, and the figure finally recorded was the average of these two determinations. The correspondence between the two tubes was generally close. From 150 cases for each sugar we have calculated the probable error of a single observation, and find it only ± 0.043 for dextrose and ± 0.036 for lactose. Since our readings were only taken to 10ths of a c.c., it is evident that even a single determination would be sufficiently accurate for any long series.

The general results of the titrations are shown in Table 25 and in Fig. 2. It will be noticed that with both acids the organisms are ranged with fair regularity about a single mode. The majority of the cultures studied produce an acidity of 0.1–0.2 per cent normal in dextrose, and fail to ferment lactose at all. In either case a few cultures only show an alkaline reaction, and with lactose less than half of the organisms form acid, giving the curve for that sugar a very acute form. The curve for dextrose falls off much more slowly, and shows slight secondary modes at acidities of 0.5–0.6 per cent normal and 1.3–1.4 per cent normal. Finally, both curves show an extraordinary extension in the direction of the higher acidities. It will be noticed that for each sugar several of the highest reactions, ranging from 3 to nearly 10 per cent normal, have been omitted from the chart. We may fairly consider the fermentation of the two carbohydrates together, since, as shown in Table 26, they are very closely correlated. The amount of acid produced in lactose broth is almost always less than that in dextrose broth, but the two vary together.

TABLE 25.

ACID PRODUCTION IN SUGAR BROTHS.

Acidity Produced (Per Cent Normal)	Number of Cultures in Each Group							
	−0.9–0.8	−0.7–0.6	−0.5–0.4	−0.3–0.2	−0.1–0.0	0.1–0.2	0.3–0.4	0.5–0.6
Lactose	1	1	7	41	204	86	52	44
Dextrose	..	..	4	9	98	110	80	76

	0.7–0.8	0.9–1.0	1.1–1.2	1.3–1.4	1.5–1.6	1.7–1.8	1.9–2.0	2.1–2.2	2.3–2.4
Lactose	23	10	7	4	5	2	1	3	2
Dextrose	45	28	12	13	6	2	0	4	1

	2.5–2.6	2.7–2.8	2.9–3.0	3.1–3.2	3.3	4.0	4.3	4.6	4.7	5.9	8.2	8.6	9.7
Lactose	1	1	..	..	1	..	2	1	..	1	..	..	..
Dextrose	3	2	0	2	..	1	..	..	1	..	1	1	1

TABLE 26.

CORRELATION BETWEEN FERMENTATION OF DEXTROSE AND LACTOSE BROTHS.

Dextrose Acid Production (Per Cent Normal)	Lactose −0.2 and more alkaline	Lactose −0.1 and 0	Lactose 0.1–0.4	Lactose 0.5–1.4	Lactose 1.5 and over
0 and alkaline	19	72	15	4	1
0.1 and 0.2	11	57	29	13	0
0.3–0.6	11	47	55	40	1
0.7–2.0	9	26	36	29	6
2.0 and over	0	2	1	2	12

We have noted before that the power of carbohydrate fermentation is specially characteristic of the parasitic cocci, and of those which do not show the packet grouping. The high acidities are also correlated with a positive reaction to the Gram stain and with a faint surface growth on artificial media.

TABLE 27.

CORRELATION BETWEEN FERMENTATION OF DEXTROSE BROTH AND NITRATE REDUCTION.

Dextrose Acid Production (Per Cent Normal)	No Reduction	Nitrite Found	Ammonia Found
0 and alkaline	75	13	16
0.1 and 0.2	86	11	16
0.3–0.6	115	28	18
0.7–2.0	90	18	15
2.0 and over	16	0	0

Table 27, for the relation between nitrate reduction and acid formation in dextrose broth, shows only that the class of strong

acid-formers fail entirely to form nitrites and ammonia. A correlation table for lactose, which we have not thought it necessary to quote here, shows a similar relation. The high acid-formers, it may be remembered, belong to the genus *Streptococcus*, with its weak power of growth on artificial media.

Our tables of the correlation between carbohydrate fermentation and optimum temperature fail to show any striking coincidences. There is an appreciable tendency for the higher acid-formers to grow better at 37°, and for the alkaline or neutral forms to grow better at 20°; but we have not considered this important enough to warrant the reproduction of the tables.

TABLE 28.

CORRELATION BETWEEN GELATIN LIQUEFACTION AND FERMENTATION OF DEXTROSE BROTH.

Acid Production (Per Cent Normal)	Gelatin Not Liquefied	Gelatin Liquefied (0.1–1.5 cm.)	Gelatin Liquefied (1.6 cm. and over)
0 and alkaline.....	38	61	12
0.1 and 0.2.....	45	42	23
0.3–0.6.....	47	37	72
0.7–2.0.....	43	36	27
2.0 and over.....	13	1	3

TABLE 29.

CORRELATION BETWEEN GELATIN LIQUEFACTION AND FERMENTATION OF LACTOSE BROTH.

Acid Production (Per Cent Normal)	Gelatin Not Liquefied	Gelatin Liquefied (0.1–1.4 cm.)	Gelatin Liquefied (1.5 cm. and over)
-0.2 and more alkaline	20	19	11
-0.1 and 0.....	91	86	27
0.1–0.4.....	49	46	43
0.5–1.4.....	13	25	50
1.5 and over.....	13	1	6

The relation between the organisms which ferment the sugar broths and liquefy gelatin is shown in Tables 28 and 29. These tables may be considered together, as they reveal practically the same law. The relation between acid production and gelatin liquefaction is evidently a somewhat complex one. The forms which fail to ferment carbohydrates for the most part exhibit a moderate amount of liquefaction. Next comes a group of the moderate acid-producers which liquefy most actively. Finally, the highest acid-formers are mostly non-liquefiers. We shall get more light on these three groups when we come later to consider the classes of the cocci according to their chromogenesis.

To our conception of the non-acid-forming cocci as typically saprophytic organisms frequently occurring in packets and usually Gram-negative, we may add the property of moderate, but not very active, liquefaction of gelatin. The very high acid-producers are generally parasitic, do not show sarcinæ, stain by Gram, grow faintly on agar, and fail to reduce nitrates or liquefy gelatin. Between these two groups is a third type which forms a moderate amount of acid and produces the most active liquefaction of gelatin.

6. Reduction of Nitrates.

As described above, the tests for nitrate reduction were made in parallel in 10 tubes, and a marked variation was found in the individual tubes, as shown in Table 30. This is perhaps to be expected, since the development of bacteria in such a nutrient medium as nitrate solution must be subject to many chance variations in the number and vigor of the organisms inoculated.

TABLE 30.

Reduction of Nitrates.

Number of Tubes Showing Positive Tests.....	1	2	3	4	5	6	7	8	9	10
Nitrites.....	27	14	8	5	11	5	11	7	12	24
Ammonia.....	30	26	21	15	16	10	2	11	4	22

Table 30 shows a considerable number of cultures yielding positive results in one or two out of the 10 tubes tested, less in from four to seven of the tubes, and more again giving check results in all 10 tubes. In order to compare this property with others, it was necessary to distinguish between positive and negative cultures, and we have therefore considered the test to be positive when five or more of the tubes showed some reduction. The cultures then grouped themselves into three classes—one a large one, of those organisms which did not have the property of nitrate reduction, and the two smaller classes, in which were those which formed nitrites and those which formed ammonia.

Table 31 shows a somewhat surprising lack of correlation between the formation of the two reduction products for which we have miad estt Only 17 cultures showed both nitrites and ammona in efives

or more of the 10 tubes, while 48 cultures formed nitrites alone, and 53 cultures ammonia alone, according to the same standard. It seems improbable that in the latter case nitrites had been formed and entirely reduced to ammonia. We are inclined rather to conclude

TABLE 31.

CORRELATION BETWEEN NITRITE FORMATION AND AMMONIA FORMATION.

	Nitrites +	Nitrites −
Ammonia +	17	48
Ammonia −	53	382

that two different types of reduction exist, in one of which ammonia is produced directly.

As regards correlation with other properties, we have seen that the production of nitrites, and still more notably that of ammonia, is especially characteristic of the cocci isolated from the air. It is, of course, possible that this may be indirectly connected with the fact that forms which have survived drying and dispersal through the air must be particularly well adapted to conditions which obtain in the nitrate solution. A similar law is apparently manifest in the striking relation to vigor of surface growth. The power of forming both reduction products increases progressively with the richness of surface growth, being entirely absent in the "very faint" class. No relation appears between nitrate reduction and optimum temperature, and the only other correlation to be considered is that with gelatin liquefaction shown in Table 32.

TABLE 32.

CORRELATION BETWEEN NITRATE REDUCTION AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (in cm.)	Nitrates Not Reduced	Nitrites Formed	Ammonia Formed
0.0	152	26	13
1.-1.5	131	27	25
1.6 and over	99	17	27

Table 32 shows the usual large proportion of organisms which do not exert nitrate reduction, but it may be noticed that some 30 per cent of the liquefiers reduce nitrates, against only 20 per cent of the non-liquefiers. Again, only 6 per cent of the non-liquefiers, against 16 per cent of the liquefiers, form ammonia.

7. OPTIMUM TEMPERATURE.

We have divided the cocci into five groups, according to their optimum growth temperature. Forty-one cultures gave "much better," and 42 "better," growth at 20°; 335 developed "equally" at both temperatures; 57 grew "better," and 25 "much better," at 37°.

We have classed together the first two and last two groups. In making the tables it was more convenient to have fewer groups, and quite as accurate, since the main distinctions (and those not very rigid) are shown in "better growth at 20°" or "better at 37°," and "equal" growth at both temperatures.

We have already noted the correlation between optimum temperature and habitat, the parasitic forms growing best at 37°, and the saprophytic forms at 20°, when any difference appears. The sarcinæ belong notably to the second class, as do the Gram-positive cultures. These are the only correlations which have so far appeared.

We have been somewhat surprised not to find special correlation between the optimum temperature for growth and that for color production; but no such correlation appears. With gelatin liquefaction also no definite relation appears.

We have observed also the effect of the body and room temperature upon color production, but without important results. Of the cocci studied, 69 showed a very much higher chromogenic power at 20° than at 37°; 169 showed more color, but not so much more, at the lower temperature; in 245 cases no difference appeared, while 13 cultures showed more, and 14 cultures much more, pigment at 37°. We have calculated correlation tables for all the various characters studied, but in no case did any constant relation appear, except, as noted later, in connection with the kind of chromogenesis.

8. CHROMOGENESIS.

As noted above, chromogenesis was determined by matching the pigment dried on white paper against a color chart prepared after a thorough study of the colors actually found among the cocci. This chart included nine hues, designated by Roman numerals, corresponding to the pigments noted below the figure. Under

each hue were nine different chromas, indicated by Arabic numerals, each figure indicating the number of washes of pure color added to obtain the particular chroma.

The distribution of the organisms studied under these different colors is indicated in Fig. 3, where the vertical columns indicate the hues from I (white), through the yellows (II–IV), the oranges (V and VI) to the reds (VII–IX), and the horizontal columns the successive chromas.

On inspection of this chart, bearing in mind the colors signified, there appear at once four modes – one occurring in each chief color.

That for the white falls at I_1, for the yellows at IV_3, for the oranges at VI_8, and for the reds at VII_8. These are not, of course, the points of intensest color, but of the most concentrated distribution. The evident clustering of the individuals around a mode, and the consequent falling-away of the numbers between the modes, suggest a variation from an ancestral center. Like most living things governed by an evolutionary law of gradual change, the hues grade so gently into each other that the exact placing of lines of division must be arbitrary. We have, however, assumed four divisions as a basis for our work, and separated them at the lowest points between the modes, as shown by the heavy black lines in the chart, which divide the group of bacteria producing a white pigment from that which produces a yellow, the yellow from the orange, and the orange from the red. The striking correlations obtained between chromogenesis and other properties have convinced us that this division was a sound and natural one. It should be noted, however, that the division of the "white" chromogens includes two subgroups—the true white pigment-formers and the forms which produce such a faint surface growth that no distinct color is apparent.

We have omitted the consideration of chromogenesis from our correlation tables, except that for habitat, preferring to consider all chromogenic relations under one head. It will appear, on inspection of the following tables, that this character is really the key by which most of the other correlations may be explained, and is perhaps the most important single factor in the systematic grouping of the Coccaceæ.

It was shown under "Habitat" that the white and orange chromo-

gens were chiefly parasites, the yellow and red chromogens chiefly saprophytic forms. The same distinction is shown in Table 33 with regard to cell-grouping. The white and orange cocci only rarely,

TABLE 33.

CORRELATION BETWEEN CHROMOGENESIS AND CELL-GROUPING.

Cell-Grouping	White	Yellow	Orange	Red
Irregular Groups and Chains	33	134	163	15
Packets	7	120	18	10

the latter very rarely, show packets. The yellow and red forms, on the other hand, show the sarcinæ-grouping almost half the time.

TABLE 34.

CORRELATION BETWEEN CHROMOGENESIS AND GRAM STAIN.

Gram Stain	White	Yellow	Orange	Red
−	6	109	15	15
±	9	84	46	7
+	25	61	120	3

The reaction to the Gram stain exhibits a still more perfect correlation. Among the whites and oranges (the parasitic forms) positive Gram reactions predominate, and negative ones are rare. Among the saprophytic yellows and reds conditions are symmetrically reversed.

TABLE 35.

CORRELATION BETWEEN CHROMOGENESIS AND SURFACE GROWTH.

Surface Growth	White	Yellow	Orange	Red
Very faint	14	3	2	0
Meager	3	6	12	0
Good	7	100	107	1
Abundant	13	95	58	24
Very heavy	3	50	2	0

A comparison of the general vigor of growth shows that each color has its own relation. Among the white forms, two maxima appear, one under "very faint" growth and one under "abundant" growth. This is because this group is a compound one, including forms which give a really white growth abundant in amount, and the feebly growing streptococci which are classed here, although they produce no pigment at all. The yellow and orange chromo-

gens show maxima under the "good" growth, almost all the "very abundant" growths belonging to the former class. The red forms are almost all of one type—the "abundant."

TABLE 36.

CORRELATION BETWEEN CHROMOGENESIS AND DEXTROSE FERMENTATION.

Acid Produced (Per Cent Normal)	White	Yellow	Orange	Red
0.0 and alkaline	5	94	7	5
0.1–0.2	7	72	24	7
0.3–0.6	5	50	92	9
0.7–2.0	15	35	53	3
Over 2.0	8	3	5	1

TABLE 37.

CORRELATION BETWEEN CHROMOGENESIS AND LACTOSE FERMENTATION.

Acid Produced (Per Cent Normal)	White	Yellow	Orange	Red
−0.2 and more alkaline	3	33	9	5
−0.1 and 0.0	8	141	39	16
0.1–0.4	12	59	64	3
0.5–1.4	6	18	63	1
1.5 and over	11	3	6	0

The correlations between chromogenesis and the fermentation of the sugars are singularly perfect. The white forms in each case show two maxima, one corresponding to the true white chromogens, the second, at a higher acidity, to the colorless streptococci. The latter include a majority of the strongest acid-producers in each case. The other types show for each sugar a regular and characteristic curve, the elements from which the complex curve in Fig. 2 is made. The yellow forms show for each sugar a mode at the neutral point. The orange chromogens, on the other hand, are most abundant at an intermediate grade of acidity, most of them producing 0.3–0.6 per cent acidity in dextrose broth, and 0.1–0.4 per cent in lactose broth. The red forms show the same relation as the orange forms toward dextrose, while in lactose broth they resemble the yellow chromogens, producing in most cases no change of reaction.

TABLE 38.

CORRELATION BETWEEN CHROMOGENESIS AND NITRATE REDUCTION.

	White	Yellow	Orange	Red
No reduction	35	197	137	13
Nitrites produced	3	30	25	12
Ammonia produced	2	37	26	0

With regard to the reduction of nitrates, the white and colorless forms show generally negative results. Nitrites are produced by one in 10 of the yellows, a slightly higher fraction of the orange forms, and by half the red-pigment-producers. Ammonia production, on the other hand, appears in one in eight of the yellows, one in 10 of the orange forms, and not at all among the reds.

TABLE 39.

CORRELATION BETWEEN CHROMOGENESIS AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	White	Yellow	Orange	Red
20°	4	66	13	0
20° or 37°	28	156	126	25
37°	8	32	42	0

Excluding the majority of forms which grow equally at either temperature, it appears that, among the white and orange forms, most of those which exhibit any preference grow best at 37°, while among the yellows 20° is more often the optimum. These results accord with the habitats, respectively parasitic and saprophytic, of the two classes.

TABLE 40.

CORRELATION BETWEEN CHROMOGENESIS AND OPTIMUM TEMPERATURE FOR COLOR FORMATION.

Color Production	White	Yellow	Orange	Red
Better at 20°	2	90	125	21
Equal at 20° and 37°	38	164	56	4

It appears from Table 40 that temperature differences affect the production of orange pigment much more than that of yellow and that the body temperature interferes with red chromogenesis most of all.

TABLE 41.

CORRELATION BETWEEN CHROMOGENESIS AND GELATIN LIQUEFACTION.

Gelatin Liquefaction, cm.	White	Yellow	Orange	Red
0.0	27	83	55	21
0.1–1.5	8	126	39	4
1.6 and over	5	45	87	0

The liquefaction of gelatin presents another close correlation with pigment production. The white and red forms are almost all non-liquefiers, the yellow cocci show a maximum among the

moderate liquefiers, and the orange chromogens exhibit the peptonizing power to a high degree.

To sum up, the cocci show four (or five) distinct groups according to their pigment production, each group being marked by a number of other correlated characters. The "white" forms rarely show packets, generally stain by Gram, fail to reduce nitrates, grow well at 37°, and usually fail to liquefy gelatin. They include two subgroups—the feebly growing, strongly acid-producing forms, which are really colorless, not white, and the white-pigment-producers, which grow abundantly and produce only a slight amount of acid. The "yellow" chromogens frequently show packets, are usually Gram-negative, give a good to a very heavy surface growth, produce little or no acid, occasionally form nitrites or ammonia in nitrate solution, grow well at 20°, and show a moderate liquefaction of gelatin. The "orange"-pigment-formers are very rarely packets, stain well by Gram, form good surface growths, produce a moderate acidity in sugar broth, occasionally reduce nitrates to nitrites or ammonia, grow well, but with poor pigment production, at 37°, and generally produce a considerable liquefaction of gelatin. The red-pigment-producers are often packets, generally Gram-negative, grow abundantly, ferment dextrose but not lactose, form nitrites, but not ammonia, in nitrate solution, grow well at 20° or 37°, producing less pigment in the latter case, and generally fail to liquefy gelatin.

9. GELATIN LIQUEFACTION.

In the routine determination of gelatin liquefaction we have used only one tube for each culture. Duplicate determinations were made on 79 cultures, from which it appeared that the probable error of a single observation is only ±0.12 cm.; so that our method was sufficiently accurate.

Of the cultures observed, 186 failed to liquefy gelatin, and the distribution of the other 314 cultures according to the amount of

TABLE 42.

GELATIN LIQUEFACTION.

Depth in cm.	0.1–0.5	0.6–1	1.1–1.5	1.6–2.0	2.1–2.5	2.6–3.0	3.1–3.5	over 3.5
Number of cultures	33	76	68	48	44	29	13	3

liquefaction after four weeks is shown in Table 42. Fig. 4 shows graphically the skew curve plotted from these data.

There is, as would be expected, a gradual falling-away toward the highest amounts of liquefaction, and the abrupt downward falling of the curve toward the non-liquefiers at 0 is extremely significant as indicating a sharp difference between the two groups. If it had been practicable to plot the non-liquefiers on this figure, the curve would have gone up at an acute angle more than twice as high as that of the mode of the liquefiers. This angle divides with more than usual definiteness those organisms which liquefy from those which do not liquefy gelatin.

The correlations of gelatin liquefaction with other properties have been already considered. We have found that a high pepton izing power is rare among the earth and water cocci and the sarcinæ. It is most frequently associated with the smaller individual cells among the non-packet-formers. It is absent or very rare in the cocci which show only faint surface growths. Finally, it appears that the white, colorless forms which produce high acidities, as well as the red chromogens, are non-liquefiers. The yellow cocci which produce little acid are moderately active liquefiers, and the orange forms with a moderate acid production show the highest peptonizing power.

IV. CONCLUSIONS FROM THE INVESTIGATION.

1. FOUNDATION OF SUBFAMILIES AND GENERA AMONG THE COCCI.

The extreme variability of the cocci has appeared with great clearness in the present study. Almost every one of the characters measured shows a wide range of fluctuations. In view of the general laws of variation, the absence of sexual reproduction, and the susceptibility of the bacteria to the direct influence of the environment, this is precisely what should be expected. It makes it, however, clearly impossible to draw sharp and arbitrary lines for any single character by which individual organisms can be naturally classified.

If, on the other hand, we examine a series of individuals with the idea of discerning central types about which they vary, the problem begins to solve itself, since such types are easily apparent. Certain organisms tend to show the packet grouping—some invariably

in every aggregate, some less constantly. Other organisms never show packets, or only very rarely. Some cocci always stain, and some always decolorize, by Gram, while intermediate forms tend more or less strongly toward either type. In surface growth two distinct types, the faint to meager and the abundant to very heavy, are manifest. In acid production there appear to be three centers of distribution corresponding to organisms which fail to ferment, those which ferment slightly, and those which produce large amounts of acid. In relation to nitrate reduction, three types appear, according as the cocci fail to reduce, or form nitrites or ammonia. On gelatin the organisms studied group themselves either as liquefiers or as non-liquefiers, and in color production four distinct centers appear, in which the pigment is white, yellow, orange, or red.

Our estimate of the value of these type-centers is greatly increased when we find that the central points for the different characters do not vary independently, but are correlated together to a remarkable degree. Again, we should expect, and we actually find, in some cases, the correlation of single characters varying, those properties generally correlated appearing in certain organisms in exceptional combinations. If, however, we consider, not the single character—not the individual organism—but the aggregate of the correlations of various properties as manifested in a considerable series of individuals, certain well-defined systematic units appear, marked by the association of a number of independent characteristics. Such an association can be explained only on the ground of relationship, and the types marked by the simultaneous occurrence of a number of properties may rightly be taken as the centers from which other, more aberrant individuals have varied.

The fact that correlation exists shows that, on the average, the fluctuations of these characters do not occur independently, but are so closely bound up with those of other properties as to vary together with them. This may be because the selective action of the environment produces a parallel change in each, or because the two characters are so closely bound together, in the physiological balance of the organism, that a change in one leads to a corresponding variation in the other. In either event it is clear that the larger systematic units (families or genera) must be marked by these pro-

found modifications of the whole center of gravity of the organism, and the smaller groups by those characters which, though perhaps showing sharper individual differences, vary by themselves without affecting any other properties.

Our object therefore has been, not to establish arbitrary boundary lines, but to discover existing natural types distinguished by the association of independent characters. In such a task it is obvious that those characters are most important which show the most marked correlations. What these characters are must be determined by study in each particular group. Chromogenesis or gelatin liquefaction may be of generic value in one family, or may mark only varieties in another, as it is, or is not, correlated with a number of other properties. In the Coccaceæ, for example, the liquefaction of gelatin and the reduction of nitrates appear, when judged by this standard, to be of less importance than most of the properties studied. In some cases they appear to be significant, but, in most of the groups indicated, liquefying and non-liquefying forms, and reducing and non-reducing forms, run parallel. Distinctions based on such a single character alone may have specific, but certainly not generic, value. On the other hand, we have been somewhat surprised to find that such apparently fluctuating characters as chromogenesis and the reaction to the Gram stain are strongly correlated with a number of other properties.

A general survey of the whole field of variation among the Coccaceæ indicates clearly the existence of two main sets of correlated characters, corresponding to the subfamilies which we have suggested in a previous communication (Winslow and Rogers, 1905). Habitat, morphology, staining reactions, surface growth, acid production, optimum temperature, and chromogenesis, all vary simultaneously in one or the other of two directions, defining the two subfamilies Paracoccaceæ and Metacoccaceæ. The first group, comprising most of the forms from the body, shows, as a rule, chains and irregular cell-grouping, stains by Gram, yields a meager or only fair surface growth, forms acid in carbohydrates, and produces no pigment, or a white or an orange one. The other group, from earth and water for the most part, often shows packets, decolorizes by Gram, grows well on artificial media, fails to ferment carbohydrates, and produces a

yellow or red pigment. It must always be remembered that each character may occasionally be found in the group where it usually does not occur; but the association of these properties in the vast majority of cases is very strong. We desire to extend our earlier definitions of the two subfamilies by including the Gram reaction and chromogenesis; and the subfamilies as thus modified will be defined at the end of this communication. It is a striking fact that these two chief divisions among the Coccaceæ correspond to the two markedly different environments which exist in nature, the body of higher organisms, and the outer world. A close correspondence with environmental conditions should naturally be expected among such simple asexual organisms as the bacteria, and it increases our confidence in the reality of the groups established below to find each of them localized so sharply in one or other of the two main environments.

Under the subfamilies we find a second grade of group-individuality, marked by the association of a smaller number of characters than the subfamilies, but still defined by the correlation of several independent properties. Here morphology, surface growth, and chromogenesis appear to be of greatest importance, acid production, gelatin liquefaction, and nitrate reduction having special significance in certain cases. Five distinct types have appeared with considerable clearness in the present study. It must be remembered that the fundamental correlations which revealed these groups were derived in an entirely impersonal way by measurements, made on each character independently, generally by different observers, and always without knowledge of the identity of the organism. When individual races are considered, it is possible, by transferring a few cultures on the border-line in a single character, to show that the correlations are really closer than have appeared above.

By this process we have attempted to group our 500 cultures under the five subdivisions suggested by the correlation tables, and have found the results so satisfactory as to confirm our confidence in their reality as natural groups. It seems to us that these groups are of such importance as to deserve generic rank. Within each there is ample room for the establishment of such a reasonable number of species as detailed study may warrant. Good genera must first be recognized, however. It is time that bacteriologists

were relieved of such vast and unwieldy and meaningless genera as now burden the science.

The first of these groups centers about a type of organism characterized by the following properties: it is parasitic in habit and grows in irregular groups, often in chains, never in packets; it stains by Gram; it grows in a thin film on the surface of agar; it ferments both lactose and dextrose with the production of a large amount of acid; it fails to reduce nitrates or liquefy gelatin, grows best at 37° and forms no appreciable amount of pigment. This corresponds to the genus *Streptococcus* (Billroth) W. and R., as previously characterized. We desire to add to our previous conception of the group the positive reaction to the Gram stain and the general failure to act on gelatin or nitrates. It must always be remembered that this genus is defined, not on morphology alone, although its members generally do show long chains in broth, but by the general complex of all its characters. Individual cultures vary from the type in some respects, as must all aggregates of organisms composed of such varying stuff as living protoplasm.

Of our 500 cultures 18 fall into the genus *Streptococcus*. All show the typical morphology (groups and long and short chains) and typical surface growth. None liquefy gelatin or reduce nitrates. Of our 18 cultures, 15 were from the body and three from polluted water. In relation to the Gram stain, 11 cultures showed positive tests, on both trials, and only two a negative test, five being variable. Of the 18 cultures, nine showed very high acidities, over 2 per cent normal, in both acids, some ranging as high as 8 to 9 per cent. The average value for the whole genus is, for dextrose 2.6 per cent, and for lactose 1.7 per cent. It is interesting to notice that those cultures which yield lower acidities are also the ones which give the negative or variable Gram reactions. Our forms therefore seem to fall into two species, 10 of them belonging to the *Str. erysipelatos* showing the very high acidities and the positive Gram reaction; the other eight differing in both these characters.

The second of our five groups is marked by a correlation of characters, of which the most obvious is the production of an orange pigment. In our previous communication we were unable to distinguish, from the literature alone, any sharp line between the orange

and yellow chromogens, and included them both under the genus *Micrococcus*. Fig. 3 makes it clear, however, that two distinct centers of variation exist, one in the orange and one in the yellow, and our correlation tables show that the two types of organisms are so radically different in every character as to demand their separation into distinct genera. Furthermore, it is evident that the orange chromogens belong with the parasitic Paracoccaceæ, and the yellow forms with the Metacoccaceæ. Nothing could show more clearly how necessary it is to make a comparative study of a large series of organisms in order to discern the true relationships of the bacteria.

For this new genus we suggest the name *Aurococcus*, as indicating the orange color, which is its most obvious characteristic. Its type-form is found on or in the plant or animal body. It occurs in groups and short chains, stains by Gram, and produces a good, but not heavy, surface growth of an orange color. It ferments dextrose and lactose, producing an acidity generally between 0.5 and 1. It grows well, but produces less pigment at 37°. It may or may not reduce nitrates and liquefy gelatin. When it does liquefy gelatin, it does so rather actively.

Of the 158 cultures in this group, all show a good, but not very abundant, growth of an orange color; 116 were obtained from the body and 30 from the air, only 12 having a saprophytic origin; 147 show groups and short chains, but no packets; and 11 occasionally give the sarcina grouping. Of the 158 cultures, 107 show a positive Gram reaction, and only nine a consistently negative one. The average acidity in dextrose for the whole group is 0.7 per cent normal, and for lactose 0.4 per cent normal. Of the 158 cultures only six form less than 0.2 per cent acid, and 17 more than 1 per cent acid, in dextrose. In lactose there is more variation; 53 cultures give less than 0.2 per cent, and 11 more than 1 per cent, acid. Of the cultures, 31 reduce nitrates, and 102 liquefy gelatin to an average depth of 2.2 cm.—a very high value; while 56 organisms fail to liquefy. The type-form of this genus is the commonest pyogenic organism, the *M. aureus* of Rosenbach. The non-liquefying forms, those which reduce nitrates, and those which produce more or less acid than is common in the genus, may later be set up as separate species.

The third of our types, like the second, has not previously been distinguished from the genus *Micrococcus;* it appears, however, to show its own definite individuality, and to belong with the Paracoccaceæ, although it approaches the saprophytic cocci in certain characters. We suggest the name *Albococcus* for this genus, which includes those organisms of which *M. pyogenes* (Ros.) Mig. is a type. They produce a more vigorous surface growth than the streptococci, with a clear white pigment, and ferment carbohydrates, producing a fair amount of acid. They are also distinguished from the Metacoccaceæ by the general tendency of their morphology and staining reactions, and by their habitat. In our series we have 23 cultures of this type. All without exception were obtained from the body or from the air, none from water or earth. All without exception show a good surface growth, white pigment, and division into groups and rarely chains, but never packets. Sixteen were uniformly Gram positive and only two uniformly Gram negative. The average acidity in dextrose broth was 0.7 per cent normal, and in lactose broth 0.5 per cent normal. Only three cultures showed an acidity lower than 0.2 per cent, and only one culture an acidity over 1.5 per cent in dextrose. Lactose results, as usual, were more variable, nine cultures falling below 0.2 per cent acid, and one above 1.5 per cent. Nitrates were reduced by three cultures and gelatin liquefied by 14. The four species which we have previously called *M. pyogenes* (Ros.) Mig., *M. rhenanus* Mig., *M. candicans* Flügge, and *M. canescens* Mig., should belong to this new genus, being distinguished, as before, by their relation to acid and gelatin. The reduction of nitrates may furnish a basis for the establishment of other species.

The fourth of the general groups which have appeared in this study is the group of the yellow pigment formers, of which *M. luteus* and *Sarcina ventriculi* are typical—a group which differs in almost all its properties from those previously considered. Organisms of this type are found mainly in earth and water rather than on or in the animal body. They give abundant, to very heavy, growths of a yellow color. They frequently occur in packets, generally decolorize by Gram, and fail to ferment sugars or ferment them only slightly. They may or may not liquefy gelatin or reduce nitrates.

Of our cultures 262 fall under this head, 195 of them coming from water, earth, or air, and only 64 from the body; 200 are uniformly or at times Gram negative, and only 62 uniformly Gram positive. The average acidity produced in dextrose broth is only 0.2 per cent normal, and in lactose broth 0.1 per cent normal. Of the 262 cultures only 33 give over 0.5 per cent acid in dextrose broth, and only 7 over 0.5 per cent acid in lactose broth; 59 of the cultures reduce nitrates; 85 fail to act on gelatin, and 177 liquefy it, producing an average liquefaction of 1.2 cm., scarcely more than half the value in the genus *Aurococcus*.

This group divides itself, according to cell-grouping, into two nearly equal divisions—those which form packets and those which do not; 136 belong to the former class, and 126 to the latter. In habitat, in Gram staining, and in relation to carbohydrates and gelatin both classes are entirely parallel. The genera *Micrococcus* and *Sarcina* are, however, so firmly established in common usage that it would require very strong evidence of identity to warrant dropping either of them. It seems best, therefore, to recognize the single character of cell-grouping as having generic value in this case, otherwise defining the two genera by the same characteristics. Under *Micrococcus* will come *M. orbicularis* Ravenel, *M. luteus* (Schröter) Cohn, and *M. ochraceus* Rosenthal; under *Sarcina*, *St. subflava* Ravenel and *S. ventriculi* Goodsir.

The fifth and last of our general types includes the sharply marked one of the red chromogens. These are entirely saprophytic forms which produce abundant surface growths of a red color. They may or may not show packets, are generally Gram negative, very rarely liquefy gelatin or ferment carbohydrates, and frequently reduce nitrates to nitrites, but apparently not to ammonia. This is the first case in which we have found the action upon nitrates markedly correlated with other characters.

Twenty-five of our cultures fall under this general type. All but one come from earth, water, or air. Only three are uniformly positive to Gram, while 15 are uniformly negative. Four of the 25 cultures liquefy gelatin, and 14 reduce nitrates to nitrites. The average acidity in dextrose broth is 0.4 per cent normal, and in lactose the average reaction is neutral. One culture in lactose and four in dextrose show an acidity over 0.5 per cent.

Here again the packets (10 in number) and the other forms (15 in number) are exactly parallel. Both show the same range of acidities and the same peculiar relation to nitrate reduction. It seems quite clear that in this case the single character of packet formation ought not to be made the basis of a distinct genus. We have recognized among the yellow forms the two genera *Micrococcus* and *Sarcina* out of deference to custom, which must always play an important part in terminology. In separating the red forms from these two old genera, however, it seems an unreasonable recognition of a false distinction to form two new ones on the single character of cell-grouping alone. We desire, therefore, to include all the red-pigment forms, characterized by the properties noted above, under one new genus, to be called *Rhodococcus*. *M. cinabareus* Flügge, *S. rosacea* Lindner, and *S. incarnata* Gruber will all belong here. Fourteen doubtful cultures are for the present omitted from this generic classification.

It remains only to summarize the characteristics of the six genera studied in this investigation in tabular form, and then to present a systematic statement of the main divisions of the Coccaceæ. It must be remembered that *Diplococcus* and *Ascococcus* have not been included in the present research and are defined solely from the the literature.

TABLE 43.

CHARACTERS OF CERTAIN GENERA OF THE COCCACEÆ.

Genus	Habitat (Per Cent Parasitic Forms*)	Cell-Grouping (Per Cent of Packet-Formers)	Gram-Stain (Per Cent of + Results)	Surface Growth	Average Acidity in Dextrose (Per Cent Normal)	Average Acidity in Lactose (Per Cent Normal)	Nitrate Reduction (Per Cent of Reducers)	Chromogenesis	Gelatin (Per Cent of Liquefiers	Liquefaction (Average Liquefaction, cm.)
Streptococcus	83	0	61	Faint	2.6	1.7	0		0	...
Aurococcus	76	7	68	Good	0.7	0.4	21	Orange.	65	2.2
Albococcus	48	0	70	Abundant	0.7	0.5	13	White..	61	1.1
Micrococcus	27	0	25	Good to Abun.	0.3	0.1	27	Yellow.	68	1.2
Sarcina	22	100	23	Good to Abun.	0.2	0.1	18	Yellow.	67	1.2
Rhodococcus	4	40	12	Abundant	0.4	0.0	56	Red...	16	0.7

*Body alone; air, source of many others. In *Albococcus* none from water or earth.

2. SYSTEMATIC SUMMARY.

Family Coccaceæ: Vegetative cells spherical.

Subfamily 1 Paracoccaceæ (Winslow and Rogers): Parasites (thriving only or best, on or in, the plant and animal body). Thrive

well under anaërobic conditions. Many forms fail to grow on artificial media; none produce very abundant surface growths. Planes of fission often parallel, producing pairs, or short or long chains, never packets. Generally stain by Gram. Produce acid in dextrose and lactose broth. Pigment, if any, white or orange.

Genus 1, *Diplococcus* (Weichselbaum): Strict parasites. Not growing, or growing very poorly, on artificial media. Cells normally in pairs surrounded by a capsule. Includes *D. pneumoniae* Weich, *D. Weichselbaumii* Trev., and *D. gonorrheae* Neisser.

Genus 2. *Streptococcus* (Billroth): Parasites (see above). Cells normally in short or long chains (under unfavorable cultural conditions, sometimes in pairs and small groups, never in large packets). Generally stain by Gram. On agar streak effused, translucent growth, often with isolated colonies. In stab culture, little surface growth. Sugars fermented with formation of large amount of acid. Generally fail to liquefy gelatin or reduce nitrates. Includes *S. erysipelatos* Fehleisen.

Genus 3, *Aurococcus*, new genus: Parasites (see above). Cells in groups and short chains, very rarely in packets. Generally stain by Gram. On agar streak good growth of orange color. Sugars fermented with formation of small amount of acid. Gelatin often liquefied, very actively. May or may not reduce nitrates. Includes *A. aureus* (Rosenbach).

Genus 4, *Albococcus*, new genus: Parasites (see above). Cells in groups and short chains (never in packets). Generally stain by Gram. Growth on agar streak abundant and porcelain white in color. Sugars fermented with production of a slight amount of acid. Gelatin liquefaction and nitrate reduction may or may not occur. Includes *A. pyogenes* (Rosenbach), *A. rhenanus* (Migula), *A. candicans* (Flügge), and *A. canescens* (Migula).

Subfamily 2, Metacoccaceæ (W. and R.): Facultative parasites or saprophytes. Thrive best under aërobic conditions. Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cell aggregates in groups, packets, or zoöglea masses. Generally decolorize by Gram. Pigment, yellow or red.

Genus 5, *Micrococcus* (Hallier): Facultative parasites or sapro-

phytes. Cells in plates or irregular masses (never in long chains or packets). Generally decolorize by Gram. Growth on agar abundant with formation of yellow pigment. Dextrose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied. Nitrates may or may not be reduced. Includes *M. orbicularis* Ravenel, *M. luteus* (Schröter) Cohn, and *M. ochraceus* Rosenthal.

Genus 6, *Sarcina* (Goodsir): Exactly like *Micrococcus*, except that division occurs under favorable conditions, in three planes, producing regular packets. Includes *S. ventriculi* Goodsir, *S. aurantiaca* Flügge, *S. subflava* Ravenel, *S. tetragena* (Mendoza) Mig.

Genus 7, *Rhodococcus*, new genus: Saprophytes. Cells in groups or regular packets. Generally decolorize by Gram. Growth on agar abundant, with formation of red pigment. Dextrose broth slightly acid, lactose broth neutral. Gelatin rarely liquefied. Nitrates generally reduced to nitrites, but not to ammonia. Includes *R. cinnabareus*, Flügge, *R. roseus* Flügge, *R. fulvus* Cohn, *R. agilis* (Ali Cohen), *R. rosaceus* Lindner, and *R. incarnatus* Gruber.

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